Chemo-enzymatic synthesis of mimics of cyclic adenosine 5'-diphosphate ribose

Bailey, Victoria Clare

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CHEMO-ENZYMATIC SYNTHESIS
OF MIMICS OF
CYCLIC ADENOSINE 5'-DIPHOSPHATE RIBOSE

A Thesis Submitted by Victoria Clare Bailey

for the degree of Ph.D. of the University of Bath 1997

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ABSTRACT

Cyclic adenosine 5′-diphosphate ribose (cADPR) is a naturally occurring analogue of nicotinamide adenine dinucleotide (NAD^+) which has been shown to release intracellular Ca^{2+} from a wide variety of cells. Interest in this novel metabolite was first aroused when it was shown to be more effective than, and work independently of, inositol (1,4,5)-trisphosphate and it is thought that this molecule may be the endogenous regulator of the ryanodine-sensitive Ca^{2+} channels. As the ryanodine receptor binding site for cADPR is unknown a way to probe the pharmacology of this Ca^{2+}-release mechanism is to build up a structure activity profile.

To date there is no total chemical synthesis of cADPR, or its analogues, so this thesis favoured a chemo-enzymatic approach. Analogues of NAD^+ were chemically synthesised and cyclised using the natural enzyme, *Aplysia* ADP-ribosyl cyclase, to give structurally modified analogues of cADPR. In order to maximise the range of synthetic analogues a route was developed such that any modified adenosine could be synthesised, selectively phosphorylated and coupled to nicotinamide mononucleotide (NMN) to give an analogue of NAD^+. Several methods for achieving each of these chemical steps are discussed. These NAD^+ analogues were then cyclised enzymatically to give modified cADPR. Although the enzyme had a loose substrate specificity, both the nucleophilicity of the purine N1 atom and substitution of the NMN ribose modified its action. Additionally the design and synthesis of inhibitors of this cyclase was initiated with the synthesis of nicotinamide benzamide dinucleotide which was a potent competitive inhibitor.

The pharmacological activity of the novel analogues were tested in sea urchin egg homogenate. To further investigate the known result that modification at the 8-position leads to antagonists, the N7 atom was replaced with a -CH- which led to the first partial agonist (7-deaza-cADPR). This analogue also had increased hydrolytic stability over cADPR due to the modified properties of the purine ring. When this modification was combined with a substitution at the 8-position the first hydrolytically stable antagonist was synthesised (7-deaza-8-bromo-cADPR). This novel analogue also had the advantage of being membrane permeable. Modifications were also made to the adenosine ribose ring. Replacement of the ribose ring with a cyclopentyl ring gave a second analogue with increased stability against enzymatic hydrolysis (cArisDPR) and a study of a series of adenosine ribose modified analogues showed that the conformation of this ribose ring was crucial to the activity of cADPR.

PUBLICATIONS

Cyclic aristeromycin diphosphate ribose: a potent and poorly hydrolysable Ca^{2+}-mobilising mimic of cyclic adenosine diphosphate ribose. V. C. Bailey, S. M. Fortt, R. J. Summerhill, A. Galione and B. V. L. Potter, FEBS Letters, **379**, 227-330, 1996.

7-Deaza cyclic adenosine 5′-diphosphate ribose: first example of a Ca^{2+}-mobilizing partial agonist related to cyclic adenosine 5′-diphosphate ribose. V. C. Bailey, J. K. Sethi, S. M. Fortt, A. Galione and B. V. L. Potter, Chemistry and Biology, **4**, 51-61, 1997.

Synthesis of 7-deaza-8-bromo-cyclic adenosine 5′-diphosphate ribose: first hydrolysis resistant antagonist at the cyclic adenosine 5′-diphosphate ribose receptor. V. C. Bailey, J. K. Sethi, A. Galione and B. V. L. Potter, J. Chem. Soc., Chem. Commun., in press.

7-Deaza-8-bromo-cyclic ADP ribose the first membrane permeable, hydrolysis resistant cyclic ADP ribose antagonist. J. K. Sethi, R. M. Empson, V. C. Bailey, B. V. L. Potter and A. Galione, J. Biol. Chem., submitted.

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QUOTATIONS

"It has long been the writer's belief that the methods used by the living organism owe their air of magic largely to our lack of knowledge of the simple chemistry of the esters of phosphoric and pyrophosphoric acid."

Sir Alexander Todd

"Our problems are the tools which God uses to polish us, not to demolish us."

Anon

ABBREVIATIONS

Adenophostin A	3-*O*-(α-D-glucopyranosyl)-adenosine 2', 3", 4"-trisphosphate
ADP	adenosine 5′-diphosphate
ADPR	adenosine 5′-diphosphate ribose
AMP	adenosine 5′-monophosphate
ATP	adenosine 5′-triphosphate
Ca^{2+}	calcium ions
cADPR	cyclic adenosine 5′-diphosphate ribose
cADPRP	cyclic adenosine 5′-diphosphate ribose 2′-phosphate
CaM	calmodulin
cAMP	cyclic adenosine 5′-monophosphate
$CaSO_4$	calcium sulphate
CDP	cytidine 5′-diphosphate
cGMP	cyclic guanosine 5′-monophosphate
CICR	calcium induced calcium release
CNS	central nervous system
CoI	coenzyme I
CoII	coenzyme II
CO_2	carbon dioxide
DAG	diacylglycerol
DCC	dicyclohexyl carbodiimide
DCU	dicyclohexyl urea
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide

DNA	deoxyribonucleic acid
DPPC	diphenyl chlorophosphate
DPN	diphosphopyridine nucleotide
EC_{50}	concentration of entity that produces 50% of the maxiumum response
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EtOH	ethanol
GDP	guanosine 5′-diphosphate
GTP	guanosine 5′-triphosphate
h	hours
H_2	hydrogen
H_3PO_4	phosphoric acid
HEPES	2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulphonic acid
HPLC	high performance liquid chromoatography
$Ins(1,4,5)P_3$	inositol (1,4,5) trisphosphate
IP_3R	inositol (1,4,5) trisphosphate receptor
K_i	dissociation constant for an inhibitor enzyme complex
K_m	concentration of substrate at which half of the enzyme active sites are filled
MeOH	methanol
$MgCl_2$	magnesium chloride
$NAAD^+$	nicotinic acid adenine dinucleotide
NAADP	nicotinic acid adenine dinucleotide phosphate
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate

NADPH	reduced nicotinamide adenine dinucleotide phosphate
N_{am}	nicotinamide
NaH	sodium hydride
NaOH	sodium hydroxide
NMN	nicotinamide mononucleotide
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NO	nitric oxide
RNA	ribonucleic acid
RYR	ryanodine receptor
s	second
SEM	standard error of the mean
TEAB	triethylammonium bicarbonate
THF	tetrahydrofuran
TLC	thin layer chromatography
TPN	triphosphopyridine nucleotide
TsOH	*p*-toluene sulphonic acid
UDP	uridine 5′-diphosphate
UV	ultraviolet

CHAPTER ONE

INTRODUCTION

1.1 Transmembrane Signalling Systems

From the onset of the evolution of multicellular organisms it was essential that cells were able to communicate with each other in order to both co-ordinate, and regulate, their function, division and organisation. A range of extracellular signalling methods has therefore been achieved which, in mammals, has led to the sophisticated communication networks of the endocrine and paracrine systems and neurotransmission.

The mediators, hormones or neurotransmitters, are in general hydrophilic compounds which are consequentially unable to cross the target cell's lipid bilayer plasma membrane. Hence a transduction mechanism is required to couple the incoming signal to an intracelluar response. This is normally achieved by the transmitter binding to a receptor on the cell surface which activates the required internal reaction. Two exceptions are the steroid and thyroid hormones which are internalised by the cell and activate target receptor proteins directly.

Three main classes of external plasma membrane receptors have been identified.

(i) Ligand gated ion channels- the receptor consists of an oligomeric protein containing approximately 20 transmembrane segments around a central pore and is usually closed. Upon stimulation, through the binding of an agonist, the channel opens, allowing ions to rapidly flow into, or out of, the cell across a carefully regulated electrochemical gradient. Ligand binding and channel opening occur on a millisecond time scale and these receptors tend to control nervous impulses. An example is the nicotinic acetylcholine receptor [1] which, when activated by acetylcholine, causes depolarisation

of the neurone in which it is situated and the initiation of an action potential in the adjacent muscle or axon.

(ii) Tyrosine kinase linked receptors - these proteins span the membrane with the extracellular region consisting of a binding site and the intracellular region consisting of a kinase enzyme. Binding of an agonist alters the conformation of the kinase and causes pairs of receptors to dimerise. This association results in the phosphorylation of the tyrosine residues and these are then able to tightly bind to the phosphotyrosine residues of particular intracellular receptors in turn causing activation of enzymes or transcription factors. This transduction mechanism is relatively slow and it thus mediates growth factors and longer acting hormones such as insulin.[2]

(iii) G-protein coupled receptors - these receptors, the most well characterised class of receptors, are composed of seven transmembrane spanning segments connected by extracellular and intracellular loops. The third intracellular loop is longer than the others and interacts with an $\alpha\beta\gamma$ heterotrimeric G-protein which possesses GTP-ase activity. The binding of an extracellular agonist (the first messenger) stimulates the G-protein to release the α subunit which in turn releases a molecule of GDP and binds a molecule of GTP. This stimulates an effector enzyme, which is lying in the same plane of the membrane, to catalyse the release of an internal signalling molecule (or a second messenger, figure 1.1).[3] Mechanisms of this type result in considerable amplification of the initial signal, with a single agonist causing activation of the effector enzyme for a period long enough to allow the synthesis of many second messenger molecules. There are several subtypes of the G-proteins,[4] and similarly many second messengers have been identified (section 1.2).

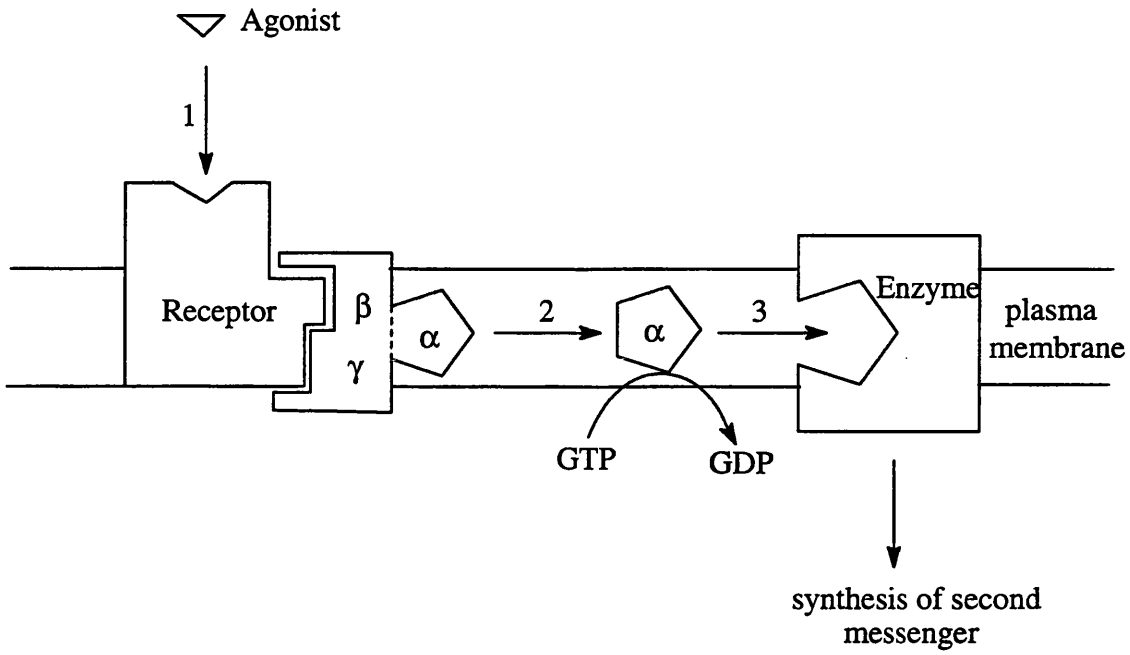


Figure 1.1 Schematic Representation of G-protein Mediated Transmembrane Signalling.

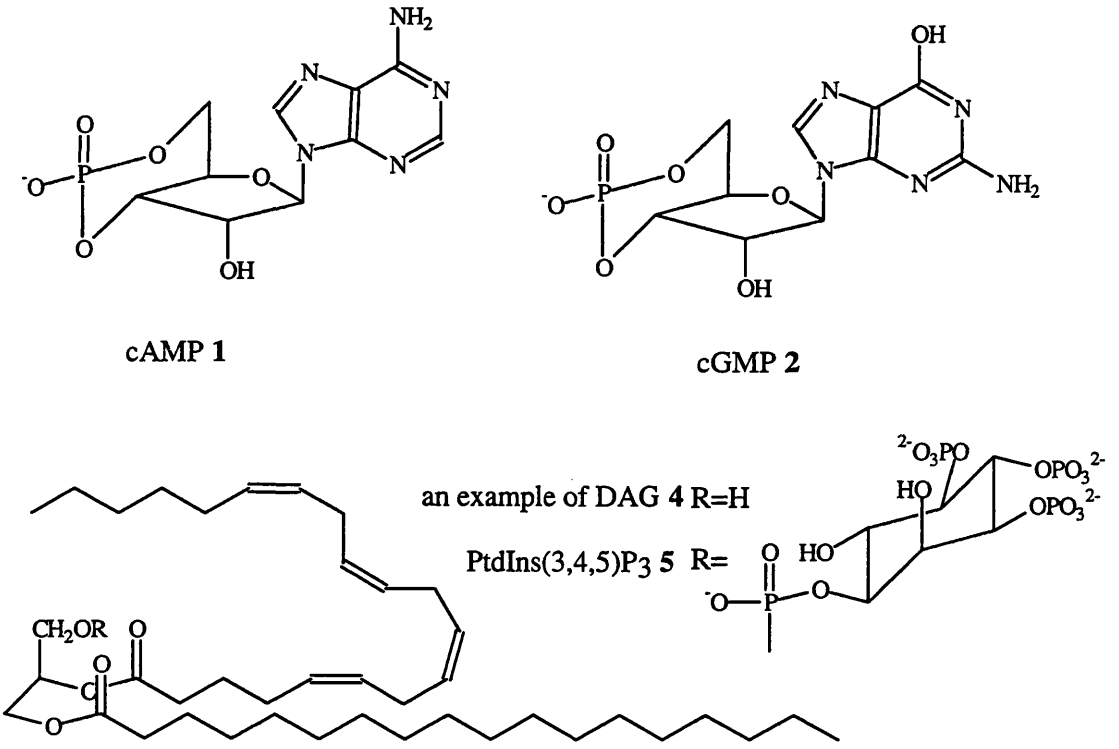
One important consequence of these signalling methods is that the same extracellular agonist is able to elicit a markedly different response in different tissues, either by (a) acting at different receptor sites, for example acetyl choline acting at the nicotinic ion channel receptor and also at the muscarinic G-protein coupled receptor;[5] and (b) by utilising different second messengers, for example agonist activation of the H_1-histaminic receptor causes production of Ins(1,4,5)P_3 whereas the H_2-histaminic receptor causes production of cAMP.[6] It is important to realise that no one of these systems operates in isolation and that in a healthy cell a delicate equilibrium exists between the various transduction mechanisms.

1.2 Second Messengers

A second messenger is an organic molecule, or more rarely a metal ion (eg Ca^{2+}) or an inorganic molecule (eg NO), acting intracellularly, whose production or release amplifies a signal such as that initiated by an agonist at the cell surface. Once the signal has been transmitted, the second messenger must be efficiently deactivated metabolically in order to terminate its action and to return the cell to its basal state.[7] Various second messengers have been identified and their pathways illucidated. These are: adenosine 3′,5′-cyclic monophosphate (cAMP, **1**) (reviewed [8]), guanosine 3′,5′-cyclic monophosphate (cGMP, **2**) (reviewed [9]) which activate protein kinases, D-*myo*-inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$, **3**) (reviewed [10]) which mobilises Ca^{2+} from intracellular stores and an example of diacylglycerol (DAG, **4**) (reviewed [11]) which activates protein kinase C. More recently, two putative second messengers have been described, namely phosphatidylinositol 3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$, **5**) (reviewed [12]) and cyclic adenosine 5-diphosphate ribose (cADPR, section 1.4). The power and versatility of such second messenger pathways is realised when it is understood that the $Ins(1,4,5)P_3$ pathway alone is coupled to at least 17 major receptor systems.[6]

1.3 The Biological Importance of Calcium

The major work of this group, of which this thesis forms a part, is concerned with the synthesis of structural mimics of three Ca^{2+} mobilising molecules - the well known second messenger molecule which causes the release of Ca^{2+} ions from internal cellular stores



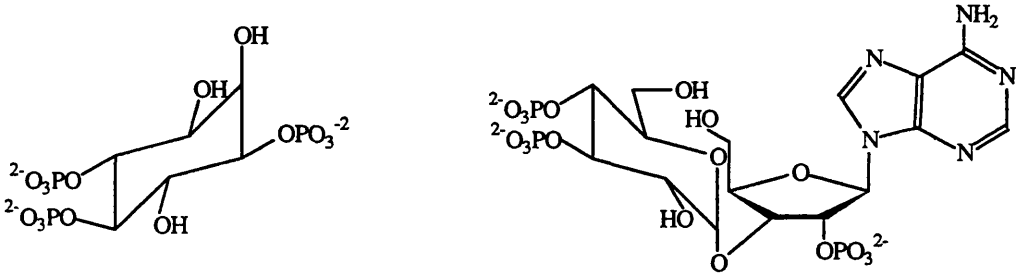


Figure 1.2 Structures of Known and Putative Second Messenger Compounds, and Adenophostin A (**6**).

$Ins(1,4,5)P_3$; the most potent agonist yet identified at the $Ins(1,4,5)P_3$ receptor, 3-*O*-(α-D-glucopyranosyl)-adenosine 2', 3", 4"-trisphosphate (adenophostin A, **6**);[13] and the putative second messenger cADPR which releases Ca^{2+} from internal stores by a different

mechanism to that of Ins(1,4,5)P_3. It is therefore of interest to include herein a brief discussion of the biological importance of Ca^{2+} to understand why its regulatory molecules are of fundamental importance to the correct functioning of living organisms.

In mammals the Ca^{2+} cation has many functions and its homeostasis is maintained by a delicate equilibrium between Ca^{2+} sequestered in bone (approximately 95%) and pools of free Ca^{2+} (the remaining 5%). These pools exist either in the external cell spaces or within the cell stored in the endoplasmic reticulum and the mitochondria. Across the store membrane exists a large concentration gradient of unbound Ca^{2+} which enables a cell to increase the internal concentration of Ca^{2+} rapidly and dramatically when required. These sharp increases of free intracellular Ca^{2+} then trigger a wide range of cellular responses depending upon the phenotype of the cell, for example, smooth muscle contraction, secretion from the exocrine gland, transmitter release from neurones and cell division and differentiation.[14]

Not only does the free Ca^{2+} cause a direct response but it can also act indirectly upon other cell functions. As the internal concentration of free Ca^{2+} rises it forms tightly bound complexes with the cytosolic protein calmodulin, changing the conformation of this protein. This complex is now able to activate various enzymes effecting other cellular responses.[15]

In some cell types, therefore, the release of Ca^{2+} may have several functions and, since each of these must be regulated independently, and may occur simultaneously, Ca^{2+} signals exhibit spatial and temporal resolution. Thus, the Ca^{2+} signal is terminated as

rapidly as it began, by reuptake by the internal stores or by pumping the ions out of the cell using active transport mechanisms. Consequently, Ca^{2+} is prevented from diffusing any distance through the cell cytoplasm, and its signal is therefore very localised. This is in contrast with other internal cell messengers which have been reported to have longer range effects.[16] A further consequence of this rapid sequestration, resulting in a low basal level of Ca^{2+}, means that inorganic phosphate, continually produced as a result of the degradation of ATP for the production of energy, can co-exist with Ca^{2+} ions without detriment. High levels of free Ca^{2+} ions would not enable this to occur since calcium phosphate would crystallise out in the cytoplasm.[17]

Calcium ions are unusual in that they are also able to trigger their own release by a positive feedback mechanism known as calcium induced calcium release (CICR), a process which is well documented in cardiac muscle. A small influx of Ca^{2+} through voltage sensitive channels triggers an explosive release of stored Ca^{2+} from the sarcoplasmic reticulum, driving the heart beat.[18] This release is believed to occur as a result of activation of internal ryanodine receptors, and may also occur in non muscle cells which express these receptors,[19] although the physiological ligand for this channel activation is unknown.[20]

Hence calcium signalling is a flexible and wide ranging mechanism by which events at the cell surface are communicated to the cell interior. The understanding of calcium release by second messengers, or otherwise, is therefore a highly important area of pharmacology.

1.4 An Introduction to cADPR

It is well understood that Ca^{2+} mobilisation from internal stores is effected by two families of closely related Ca^{2+} release channels, inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RYRs).[21] The signal transduction pathways of the IP_3R is well documented [7] but conversely little is known about the release mechanisms of the RYR although recently the importance of this second release pathway in a diverse set of cellular systems has become understood.[22] Hence, attention is being focused on gaining a clearer insight into the physiological triggers of RYRs and evidence indicates that cADPR (**7**), a recently discovered novel metabolite of β-NAD^+ (**8**) [23], may be one of the regulators.

1.4.1 Discovery of cADPR

cADPR was discovered in 1987 during a series of investigations into the mechanisms of Ca^{2+} release in sea urchin eggs.[24] This cell type had already been one of the first in which $Ins(1,4,5)P_3$ was shown to mobilise Ca^{2+} in the intact cell [25] but it was also known that other Ca^{2+} release mechanisms existed. During fertilisation, a process which involves intracellular Ca^{2+} release, it was known that cellular levels of β-NAD^+ dramatically changed.[26] The work of Clapper *et al.* [24] extended this observation by showing that, in contrast to the rapid Ca^{2+} release that is observed when $Ins(1,4,5)P_3$ is administered to the cell, there was a time lag of 1-4 minutes between the addition of β-NAD^+ and any release of Ca^{2+}. Not surprisingly they also showed that the Ca^{2+} release was stereospecific and could not be induced by addition of the α-epimer. These observations suggested that an enzymatic transformation of β-NAD^+ was occurring to form an active species which was

then triggering Ca^{2+} release. The active metabolite was at first named E·NAD^+, "enzyme activated NAD^+". It was possible to purify this active species by HPLC and analysis showed it to be a novel cyclised ADP-ribose, cADPR.[27]

1.4.2 Structure of cADPR

cADPR (**7**, figure 1.3) has, as its name suggests, a cyclic structure which is formed by linking the N1 nitrogen of the adenine ring to the terminal ribose unit. When formed from β-NAD^+ this linkage occurs with the displacement of the nicotinamide ring and with retention of the β-stereochemistry at this centre.

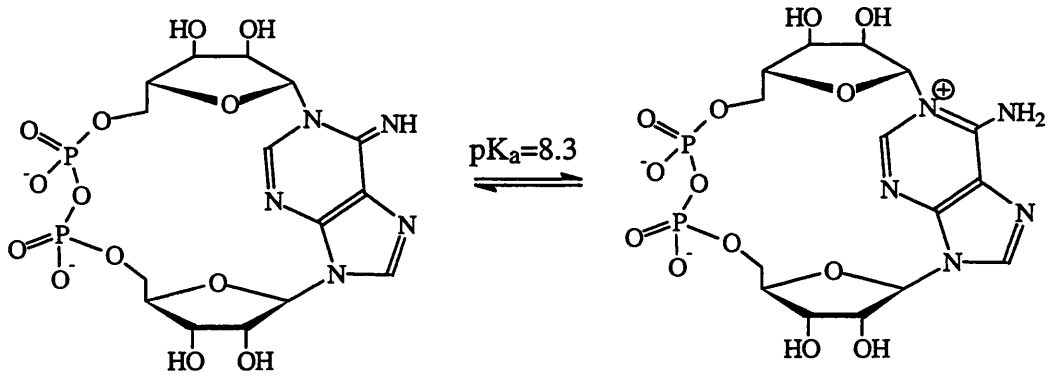


Figure 1.3 Diagram to Show the Two Different Protonated Forms of cADPR (**7**).

Several techniques were used to determine this structure from material purified from the egg homogenate by HPLC:

(i) ^1H NMR verified the removal of the nicotinamide ring and the resonance of both anomeric protons at approximately 6ppm indicated that both anomeric carbon atoms were bonded to nitrogen atoms,

(ii) mass spectrometry gave a molecular ion of exactly one water molecule less than for ADPR (**9**),

(iii) phosphate determination indicated there to be two moles of phosphate, and elemental analysis confirmed the molecular formula to be $C_{15}H_{20}N_5O_{13}P_2$.[27]

This analysis led to the proposal of a cyclic structure with an N-glycosyl linkage between the anomeric carbon of the second ribose and an adenine ring nitrogen but there were two areas of doubt. First, it was impossible to ascertain the stereochemistry of this linkage and secondly there were three possible nitrogen atoms in the adenine ring that could make this bond, N1, N7 or the amino N6. It was thought that cyclisation to a ring nitrogen would leave a positive charge on the adenine ring thus altering its UV spectrum and since the actual spectrum was similar to that for ADPR the initial structure proposed by Lee *et al.* [27] was cyclised at the amino N6. Surprisingly the authors did not record the pH or the buffer in which this UV spectrum was recorded.

In order to verify the site of cyclisation Kim *et al.* [28] carried out a detailed UV study of cADPR recording the spectra at various pH values and comparing the results to those for compounds with N-substituted adenine derivatives. It was known that at pH=11 N1 substituted adenine derivatives alone exhibited a second broad peak in the UV spectrum at 285-300nm whose appearance was reversible, in addition to the major peak at 259nm. The UV spectra of cADPR also exhibited this extra peak and therefore it was concluded that the cyclisation was at the N1 position.

The N1 cyclisation site was confirmed and the stereochemistry of the new glycosyl linkage was assigned as β with the publication of the X-ray crystal structure of cADPR in 1994.[29]

1.5 Metabolism of cADPR

cADPR is synthesised by enzyme-mediated cyclisation of β-NAD$^+$ to give the cyclic product and is catabolised by enzyme mediated hydrolysis of the N1-glycosyl linkage to give ADPR. The enzymes involved in this metabolic pathway are ADP-ribosyl cyclase and cADPR-hydrolase respectively, and their activities have been reported in many different tissues.[30] A schematic representation of the route is shown (figure 1.4).

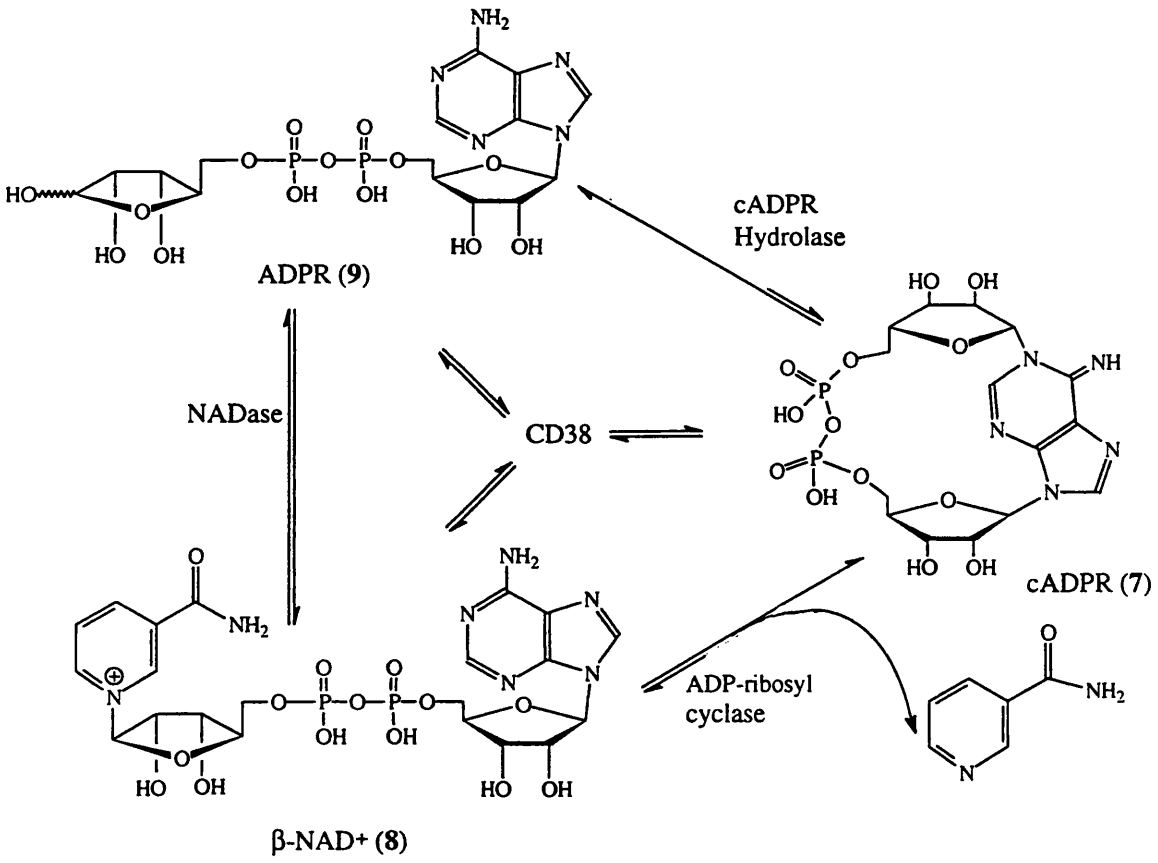


Figure 1.4 Metabolic Pathway of cADPR (7).

1.5.1 ADP-Ribosyl Cyclase

The activity of this enzyme was first observed with the discovery of cADPR itself in sea urchin egg homogenate [24] and since then its activity has been detected in a variety of mammalian tissues.[31] As with other enzymes, ADP-ribosyl cyclase is stereoselective with respect to its substrate, accepting only β-NAD^+ and not catalysing any cyclisation of the α-isomer. Its activity is sensitive to heat denaturation and protease treatment. However, in contrast with other NAD^+ utilising enzymes, ADP-ribosyl cyclase does not require any exogenous cofactors.[32]

In most tissues the activity of ADP-ribosyl cyclase is low. The enzymes are usually membrane bound and often bifunctional, also possessing cADPR-hydrolase activity, making it difficult to even observe the production of cADPR.[33] However, a 29kDa soluble form of the enzyme which has no cADPR-hydrolase activity has been purified and sequenced from the ovotestis of the marine mollusc *Aplysia californica*.[32, 34-35] This purified isoform has an unusually high specific activity of 1200unitmg^{-1} and a turnover rate of 520s^{-1}.[32] The fact that this enzyme is not bifunctional has resulted in incubation of β-NAD^+ with the *Aplysia* enzyme becoming the most widely used route for commercial cADPR synthesis.[36]

1.5.2 cADPR-Hydrolase

If cADPR is a true second messenger it must have a feedback mechanism by which its activity is halted, and therefore it was important to characterise an enzyme which

degraded cADPR. cADPR-Hydrolase has been discovered as an enzyme which can do just this.[33] It is a membrane bound protein, which has been shown to be as widespread as ADP-ribosyl cyclase and which cleaves the N1-glycosidic bond to give ADPR (**9**, figure 1.4), a linear molecule which itself is inactive as a Ca^{2+} mobilising agent.[37] Little is known about the regulation of this enzyme except that it does not appear to require any cofactors and that it may be inhibited by mM ATP levels.[38] It is of interest to note that in general cADPR hydrolases are often found associated with ADP-ribosyl cyclases on the same polypeptide [39] (with the important exception of the *Aplysia* cyclase, section 1.5.1) and this, coupled to their widespread occurrence, indicates that the cellular levels of cADPR are very tightly controlled. Recent work, which supports this conclusion, has illustrated that cADPR-hydrolase activity is inhibited by ADPR acting via a negative feedback mechanism.[40] It therefore appears that NAD^+ is converted to cADPR and then immediately, by the same protein, hydrolysed to ADPR. As the concentration of ADPR increases in the vicinity of the enzyme it acts in a feedback mechanism to inhibit the hydrolase activity and only then is cADPR able to accumulate and subsequently act as a Ca^{2+} releasing agent.

1.5.3 NAD^+ Glycohydrolases

NAD^+ glycohydrolases, more commonly referred to as NADases, have been known for more than 50 years and are classified as enzymes which convert NAD^+ (**8**) to ADPR (**9**) by hydrolysis of the nicotinamide-ribosyl linkage (figure 1.4).[41] Hence, overall, this one step process is equivalent to the two step cADPR metabolism. The NADases themselves are a diverse class of enzyme found in species from microbes to mammals, varying in size

from 20-120kDa and can be both membrane bound and soluble (reviewed [42]). However, despite this long history, any physiological functional significance remains to be established.

Although the specific roles of NADases and the enzymes involved in the metabolism of cADPR are very different, for example, commercial bacterial NADase from Sigma converts NAD^+ into ADPR with no detection of cADPR,[23] the observation that in most tissues ADP-ribosyl cyclase activity and cADPR-hydrolase activity is found on the same polypeptides is beginning to make the situation less distinct.

It is widely accepted that the mechanism of action of NADases proceeds via a covalent enzyme ADPR complex which has already lost nicotinamide (N_{am}).[42] The nucleophilic attack of this complex by a water molecule results in the hydrolysis of the enzyme covalent linkage to yield ADPR. However, if the conformation of the enzyme were such that the active site was concealed, an intramolecular attack might proceed instead to form cADPR.[39]

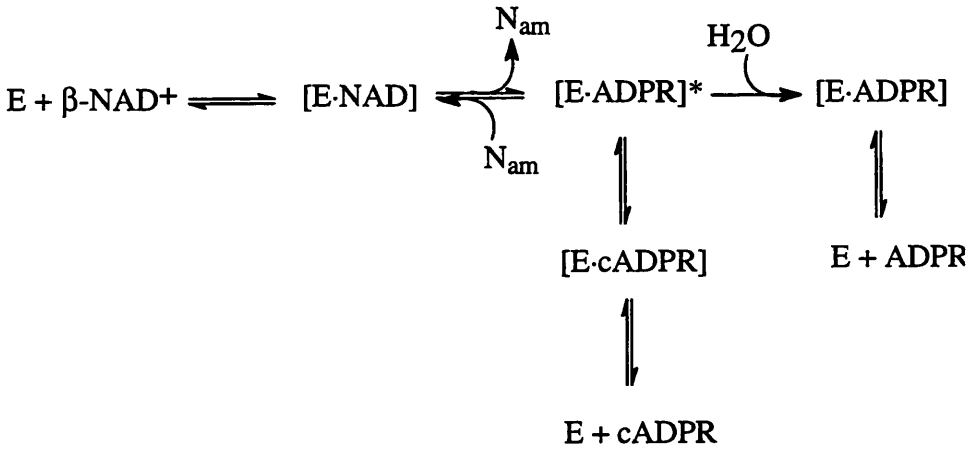


Figure 1.5 Schematic Diagram to Illustrate the Relationship Between NADases and ADP-Ribosyl Cyclase.[39]

Since ADPR and cADPR are difficult to distinguish by chromatographic methods, especially before the discovery of cADPR, many of the older studies of NADases might need to be re-examined to investigate the possibility that some of these peptides may in fact have combined ADP-ribosyl cyclase and cADPR-hydrolase activities.

1.5.4 CD38

CD38 is a surface antigen which is expressed primarily on human lymphocytes but also in other tissues and which has been implicated to play a role in lymphocyte differentiation.[43] It has been shown that this peptide possesses a high degree of sequence homology with ADP-ribosyl cyclase [44] and also that the antigen is in fact able to catalyse the conversion of NAD^+ to cADPR.[45] Additionally, this sequence has also been shown to have cADPR-hydrolase activity [46] and thus it appears that CD38 was a bifunctional enzyme apparently designed to metabolise cADPR.

The introduction of site-directed mutations to CD38 have resulted in a CD38 protein in which the cADPR-hydrolase activity has been lost and therefore only exhibits ADP-ribosyl cyclase activity. Conversely, the introduction of the opposite mutations to *Aplysia* ADP-ribosyl cyclase protein has formed a protein which exhibits not only the cyclase activity but also cADPR-hydrolase activity.[47] This work has further confirmed the close structure homology of the cyclase and hydrolase enzymes and has also highlighted those amino acid residues of particular importance for hydrolase activity.

Other membrane-bound bifunctional cyclase / hydrolase proteins have also been identified on erythrocytes,[48] bone marrow [49] and rat islets of Langerhans.[50] The discovery of each of these proteins adds further evidence to the widespread utility of cADPR. However, it is not clear whether the occurrence of the proteins on the outside of the cell corresponds to an as yet unidentified extracellular role of cADPR or whether activation of the protein leads to internalisation and intracellular release of cADPR.

1.6 Calcium Signalling and cADPR

Interest in cADPR as a Ca^{2+} mobilising agent was first aroused when it was shown to be more effective than, and to work independently of, $Ins(1,4,5)P_3$ in releasing internal stores of Ca^{2+} in sea urchin eggs.[37] Although both compounds are full agonists, cADPR was shown to be much more potent than $Ins(1,4,5)P_3$ with a half maximal effective concentration of approximately 18nM, seven times lower than that of $Ins(1,4,5)P_3$. Additionally a high dose of cADPR was shown to desensitise the microsomes to a subsequent further addition of cADPR but not of $Ins(1,4,5)P_3$, indicating that the two compounds act via different mechanisms.[37]

Further studies on the action of cADPR showed that its mechanism of Ca^{2+} release did not operate through blocking the Ca^{2+} transporting system, nor did it act as a non-specific Ca^{2+} ionophore, since it did not release Ca^{2+} from mitochondrial stores.[24] As discussed (section 1.4) there are two types of receptor on the surface of endoplasmic reticulum internal stores and the possible action of cADPR on the RYR was investigated. Cross desensitisation experiments, using the known pharmacological activators of RYR (caffeine and ryanodine) indicated that cADPR did indeed release Ca^{2+} from the same pool as these

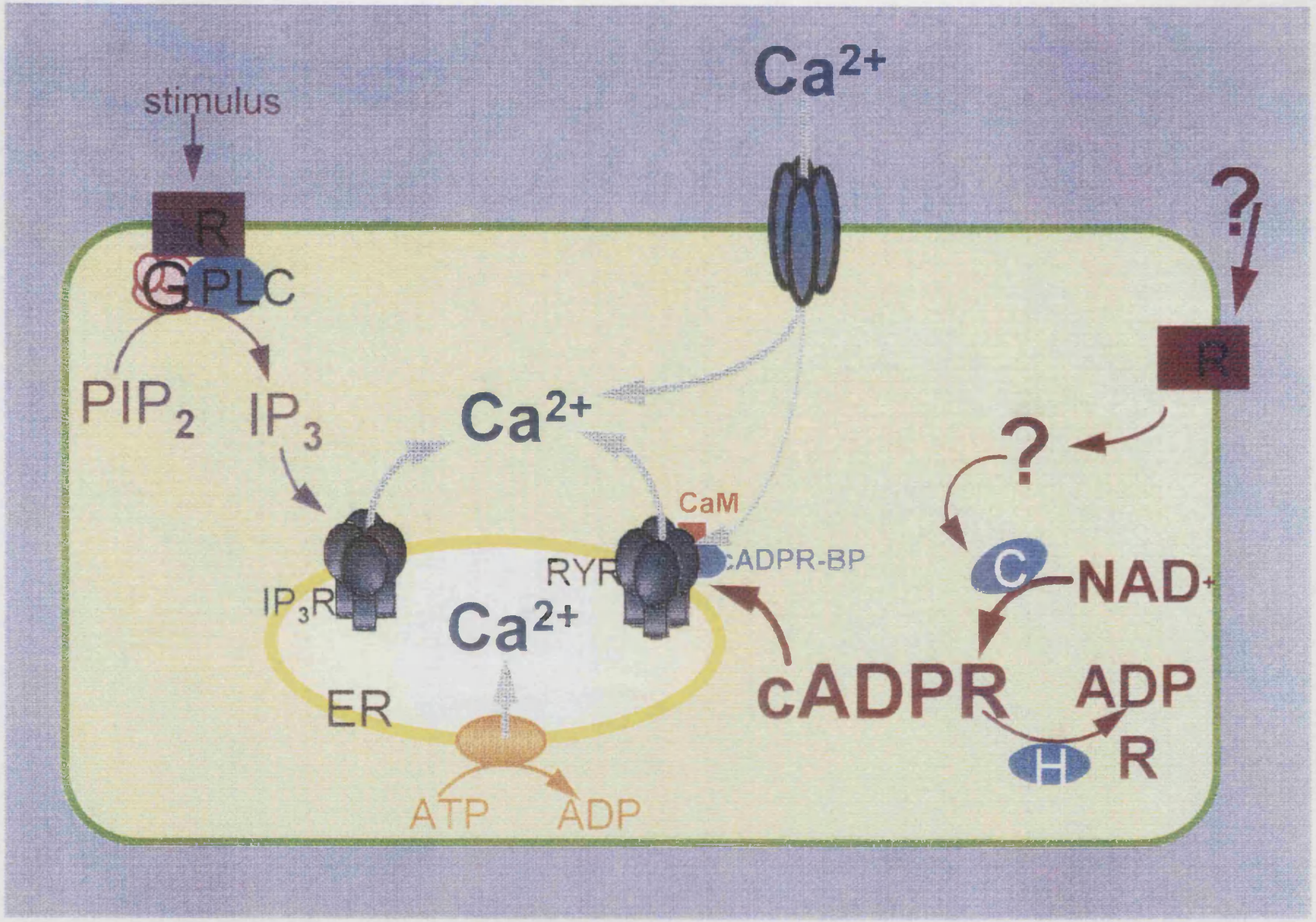


Figure 1.6 Schematic diagram to show the Ca^{2+}-release mechanisms in sea urchin eggs.

compounds. Furthermore, experiments with the classic RYR blockers (procaine and ruthenium red) inhibited cADPR mediated Ca^{2+}-release but not that induced by $Ins(1,4,5)P_3$.[51] This led to the hypothesis that cADPR is the endogenous regulator of RYRs [52] but its mechanism of action was still unclear. Figure 1.6 attempts to summarise the current understanding of the known Ca^{2+} release pathways of sea urchin eggs.

1.6.1 cADPR as a Mediator of CICR

As discussed (section 1.3) the influx of Ca^{2+} from the extracellular medium into the cell through ion channels is itself able to cause further Ca^{2+} release from the internal stores.[52] This release appears to operate via the opening of the ryanodine receptors but the mechanism is complex and appears to require the presence of a secondary factor to sensitise the receptor to the Ca^{2+} ions. Caffeine has been shown to be one of the agents able to sensitise the receptors to this release - at micromolar concentrations caffeine is able to sensitise the receptor to incoming Ca^{2+} ions, but millimolar concentrations of caffeine are required to activate the RYR in the absence of incoming Ca^{2+}.[53]

The observation that cADPR could induce Ca^{2+} release by activation of the ryanodine receptor led to further experiments to investigate whether it could also sensitise the receptor to CICR. Initially it was observed that in sea urchin eggs low concentrations of cADPR, not in themselves potent enough to elicit Ca^{2+} release, were able to sensitise the RYR to divalent cations, which then caused further Ca^{2+} release.[54] This was followed by work which showed that in bullfrog neurons cADPR was also able to potentiate the CICR signal in a manner similar to that of caffeine, and was more effective at doing so than

caffeine itself.[55] Further work has demonstrated that in sea urchin eggs this effect appears to be co-operative possibly requiring two molecules of cADPR for sensitisation.[56]

CICR is thought to be the major Ca^{2+} release mechanism of muscle cells and a property of RYR.[57] The possibility that the endogenous activator for this release is cADPR therefore has far reaching pharmacological implications.

1.6.2 cADPR as a Second Messenger

A second possible role for cADPR is that it acts to stimulate the RYR in a second messenger role analogous to that of Ins(1,4,5)P_3 upon IP_3R. For this to be the case the synthesis of cADPR must be activated by an extracellular stimulus and evidence did indeed exist to suggest that this might be true. It had been shown that the time course of the Ca^{2+} transients that were initiated at fertilisation of the sea urchin egg differed from those induced by direct microinjection of cADPR into the egg.[58] It was therefore proposed that the sperm contained a compound that initiated the synthesis of cADPR, but they did not contain cADPR itself.

One possible intriguing candidate for this initiator is cGMP (**2**, figure 1.2) which had been shown to institute a transient Ca^{2+} release similar to that seen at fertilisation when injected into sea urchin eggs.[59] Further experiments demonstrated that this action could not be blocked by the IP_3R antagonist heparin [59] but was inhibited by the RYR antagonist ruthenium red.[60] Further investigation into the pharmacology of the cGMP-induced release demonstrated the requirement of β-NAD^+, and inhibition by the cADPR antagonist

8-amino cADPR (**11**, figure 1.15).[61] In addition, analysis of the β-NAD^+ levels and their metabolites in egg homogenates treated with cGMP suggest that cGMP does stimulate the conversion of β-NAD^+ to cADPR.[60]

It is well documented that NO acts as an extracellular agonist for the intracellular production of cGMP.[62] It has been shown that NO can elevate Ca^{2+} levels both in intact eggs and in egg homogenate. The pharmacology suggests that this is via a pathway activating both cGMP and cADPR.[63] In addition it has been demonstrated that in sea urchin eggs β-NAD^+-, cGMP- and NO-induced Ca^{2+} release are all inhibited by nicotinamide. This inhibits ADP-ribosyl cyclase activity (and hence cADPR synthesis) and therefore further supports the hypothesis that cGMP and NO exert their response by stimulating cADPR synthesis.[64] Hence a possible second messenger role has been demonstrated in sea urchin eggs but no such route has yet been shown to operate in mammalian cells.

1.6.3 Calmodulin and its Effect upon cADPR Induced Calcium Release

Until recently it was assumed that cADPR alone was able to induce Ca^{2+} release from the RYR. However, it has now been shown, that when the sea urchin egg microsomes were purified by Percoll density gradient centrifugation they lost all sensitivity to cADPR and were not able to release Ca^{2+} even when challenged with a saturating concentration of cADPR.[65] The response however could be restored by the re-addition of a soluble protein factor from the supernatant. Upon purification of this sensitivity conferring factor it was found to have properties of calmodulin and addition of authentic calmodulin to the

microsomes was able to reintroduce the cADPR sensitivity[65] In addition the calmodulin inhibitor W7 was able to abolish the cADPR-sensitivity conferring activity in a concentration dependent manner.[65] Further work confirmed this factor was calmodulin.[66]

To date, the exact binding site of cADPR on RYR has yet to be identified and there is even some doubt as to whether cADPR does act directly with the receptor.[67] Interestingly though, it is known that calmodulin does have a binding site on the RYR.[68] The requirement of accessory proteins for the action of cADPR may indicate that it is a cADPR-protein complex, and not cADPR itself, which binds to the receptor. These results may also explain why cADPR activity has yet to be observed in certain cell systems.

1.7 Occurrence and Physiology of cADPR in Mammalian Cells

The above discussion describes in detail the known pharmacology of cADPR and its Ca^{2+} mobilising properties in sea urchin eggs. Following these discoveries there has been considerable interest in the activity of cADPR in other tissues and its metabolising enzymes, endogenous levels and Ca^{2+} mobilising ability have now been investigated in a wide variety of cell types. A recent, and comprehensive, review of cADPR gives a large amount of detail [23] but a few of the more interesting mammalian examples, which have direct consequences upon possible roles for cADPR, are discussed below.

1.7.1 Cardiac Muscle

Ryanodine receptors are known to be expressed in large quantities in muscle cells [22] and in cardiac muscle it is thought that CICR, mediated via these receptors, potentiates the action potential.[69] Despite this, there is no known endogenous ligand for this action and cADPR seemed to be a good candidate. Initial investigations showed both cADPR and ADP-ribosyl cyclase to be present in large quantities in rat cardiac tissues. A second study indicated that cADPR could induce Ca^{2+} release from cardiac sarcoplasmic reticulum and that this action of cADPR was blocked by ryanodine.[71] Most recently it has been demonstrated that 8-amino-cADPR (**11**, an antagonist of cADPR Ca^{2+} release) is able to suppress the excitation - contraction Ca^{2+} transients in intact cardiac myocytes of guinea pigs and is therefore suppressing the action of endogenous cADPR.[72] This evidence is consistent with the hypothesis that endogenous cADPR plays an important role in the normal contraction of cardiac cells although further work is needed in this area.

1.7.2 Pancreatic β-Cells

Glucose is the primary stimulus for the production and secretion of insulin from the pancreatic islets of Langerhans [7] and it is an increase in Ca^{2+}, from mobilisation of the Ca^{2+} from intracellular stores, that couples the two events. It had long been assumed that Ins(1,4,5)P_3 was the second messenger for this mobilisation but in some cells no turnover of Ins(1,4,5)P_3 was observed.[74] The discovery of cADPR led to its action being investigated and indeed it was shown that cADPR was able to stimulate Ca^{2+} release in these cells and also to stimulate insulin release.[75] Furthermore, increasing levels of

glucose have been shown to stimulate cADPR production [75] and the intracellular perfusion of pancreatic acinar cells with cADPR has been demonstrated to induce short-lived Ca^{2+} spikes in a dose dependent manner.[76] Following the discovery that calmodulin was required to confer sensitivity to the action of cADPR [65] it has been shown that a calmodulin dependent protein kinase II is essential for the cADPR-mediated Ca^{2+} mobilisation for insulin secretion in islets.[77] This could well explain why Islam *et al.* [78, 79] failed to observe the Ca^{2+}-mobilising effect of cADPR in their experimental system. It is now proposed that glucose acts to stimulate both the protein kinase II and cADPR production. The kinase phosphorylates the RYR thereby sensitising it to the cADPR signal which then activates Ca^{2+} release in turn causing insulin secretion (figure 1.7).[77]

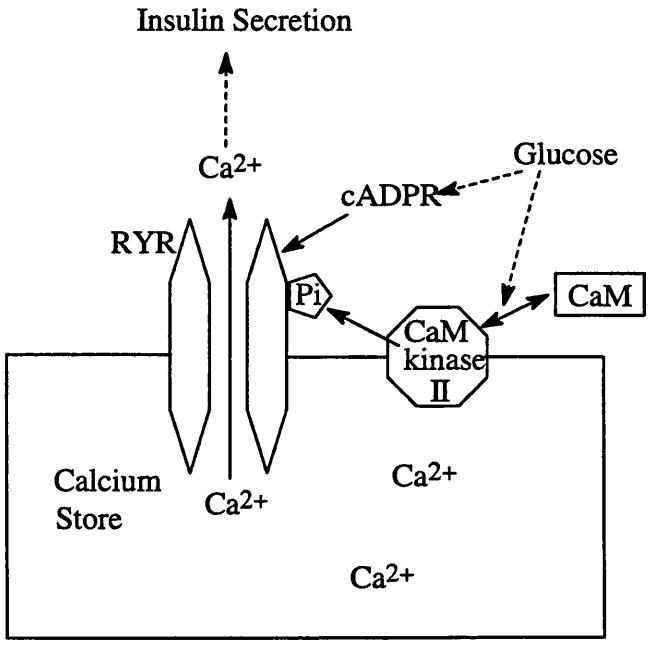


Figure 1.7 Model for the Glucose-Induced cADPR-Mediated Secretion of Insulin.[77]

CD38 has been shown to be expressed in pancreatic β-cells and recently it has been shown the Ca^{2+} mobilising activity of such cells is greatly increased when this protein is

over expressed. This work also demonstrated that ATP, a product of glucose metabolism, was able to inhibit the cADPR hydrolase activity of CD38, thus, directly allowing an increase in the concentration of cADPR in the cell. This is the first evidence that CD38 plays a regulatory role in the production of insulin.

1.7.3 T-lymphocytes

T-cells mediate the cellular immune response which destroys virus infected cells, parasites and cancer cells. The foreign antigen is recognised by the T-lymphocyte antigen receptor, a multimeric structure which is functionally coupled to the phosphatidylinositol second messenger pathway and which is in turn responsible for mobilisation of intracellular free Ca^{2+}. It is this release of Ca^{2+} which activates the cell to produce an immune response.[81] It is understood that this pathway is regulated by the release of $Ins(1,4,5)P_3$ [82] but more recently it was shown that other distinct Ca^{2+} pools were also present within the cell, one of which was emptied by caffeine which is known to activate ryanodine receptors.[83] A continuation of this work investigated whether the endogenous ligand for this release was cADPR.[84] It was shown that cADPR released Ca^{2+} from intracellular stores in T-lymphocytes and, in keeping with precedent, this release was distinct to that of $Ins(1,4,5)P_3$. The release was also shown to be inhibited by 8-amino-cADPR (**11**, figure 1.15, a known antagonist of cADPR release) and cADPR was shown to be present by direct analysis of endogenous samples by HPLC. Futhermore, preliminary studies indicated that cGMP could dose-dependently mediate Ca^{2+} release and that this release could not be further enhanced by additional cADPR, indicating that the Ca^{2+} released originated from the same stores.

1.8 A Superfamily of Pyridine Nucleotide Calcium Mobilising Agents

Following the initial discovery that Ins(1,4,5)P_3 was a potent Ca^{2+} releasing agent [85] it has become clear that metabolites of the parent compound may also have second messenger properties, leading to a potential superfamily of related inositol phosphates (reviewed [86,-87]). In a similar fashion a possible superfamily of Ca^{2+} mobilising pyridine nucleotides is emerging.

When Clapper *et al.* first investigated the calcium mobilising properties of NAD^+ in 1987 they also investigated NADP (**12**, figure 1.8), a second naturally occurring coenzyme (section 1.9.2) and showed that it too could mobilise Ca^{2+} ions.[24] Unlike NAD^+, NADP induced Ca^{2+} release without delay, indicating that the active derivative was already present in the NADP sample. Alkaline treatment of NADP greatly increased the potency of its Ca^{2+} mobilising activity and HPLC analysis confirmed that the commercial sample of NADP contained a small impurity whose concentration increased when NADP was treated in this manner. Only recently has this observation been investigated more substantially and the Ca^{2+} mobilising species purified and identified.

In 1995 Lee and Aarhus isolated the active NADP metabolite and showed it to be distinct from NADP and NADPH (**13**) by HPLC.[88] However, although high resolution mass spectrometry showed the mass to be one unit higher than that of NADP, the proton NMR was virtually identical to that of NADP. This was consistent with the active compound being nicotinic acid adenine dinucleotide phosphate (NAADP, **14**, figure 1.8).

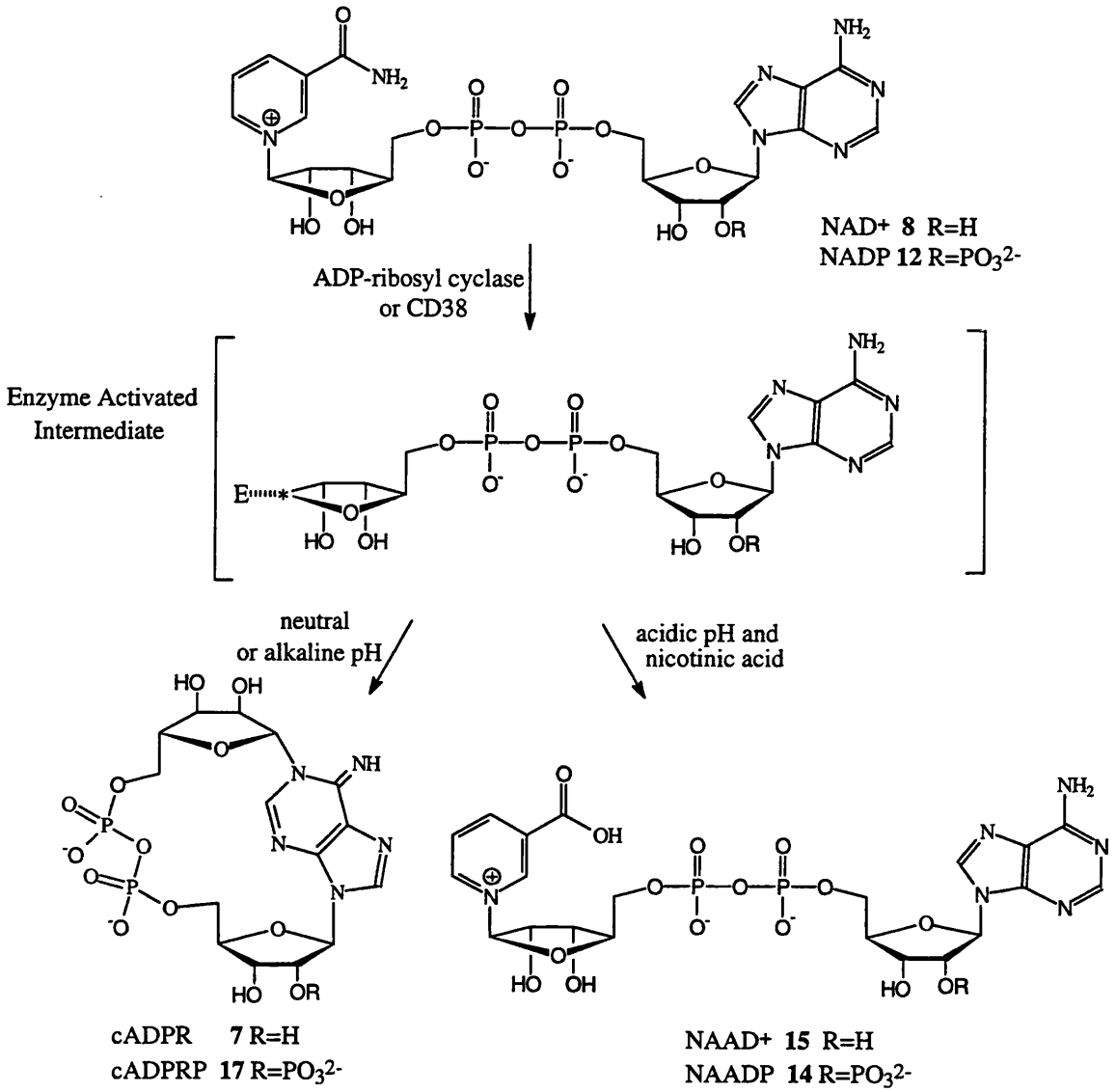


Figure 1.8 Dual Role of ADP-Ribosyl Cyclase and CD38.

Investigation of the Ca^{2+} releasing properties of NAADP (**14**) showed it to release Ca^{2+} ions with a half maximal concentration in sea urchin egg homogenate of about 30nM, similar to cADPR and more potent than $Ins(1,4,5)P_3$.[89] This release was specific, since neither $NAAD^+$ (**15**) nor NADP (**12**) could mobilise any Ca^{2+} even at concentrations 10-40 fold higher than those of NAADP. Furthermore, by desensitising microsomes to further Ca^{2+} release by NAADP, it was shown to that this agonist acts via a different mechanism

and through different stores to those activated by either cADPR or Ins(1,4,5)P_3 since subsequent addition of these agonists could release more Ca^{2+}.[90-91] Additionally, this release was insensitive to the IP_3R and cADPR antagonists heparin and 8-amino cADPR respectively. Despite acting upon different stores, the NAADP-induced Ca^{2+} release and that invoked by either cADPR or Ins(1,4,5)P_3 are non-additive, suggesting either direct or indirect communication between the different Ca^{2+} pools.[92] NAADP has also been shown to mobilise internal stores of Ca^{2+} in intact cells both by photolysis of caged NAADP [89] and by using microinjection techniques.[93] Interestingly, once fertilised, sea urchin eggs became desensitised to subsequent release of Ca^{2+} by NAADP, suggesting that this system plays a particular role during this process.

NAADP shows an unusual pharmacology in that it is a potent self-desensitiser with sub-threshold concentrations of NAADP being able to desensitise the stores to subsequent release of Ca^{2+}. Additions of concentrations as low as 1nM NAADP could desensitise the organelles to further release and a 10 minute pretreatment of the organelle with 0.25nM NAADP was sufficient to produce 50% inactivation.[89] Such a deactivation, which is thought could possibly act by altering the receptor conformation, has not been previously described.

The crucial link between NAADP release and that of cADPR came when it was shown that in the presence of nicotinic acid (**16**) and at acidic pH, both ADP-ribosyl cyclase and CD38 are able to catalyse the substitution of the nicotinamide ring of NADP (**12**) with nicotinic acid (**15**) to produce NAADP (**14**).[94] In the absence of nicotinic acid and at neutral pH the catalytic activity of the enzyme reverts to its normal behaviour and is able

to catalyse the cyclisation of NADP (**12**) to cADPRP (**17**). The cyclase is also able to synthesise $NAAD^+$ (**15**) from β-NAD^+ (**8**) under the correct conditions. It is proposed that the two different reactions of the enzyme proceed through the same activated intermediate with the first step of catalysis likely to be the release of the nicotinamide from the substrate. If nicotinic acid is present then further reaction of the activated species results in the formation of $NAAD^+$ (**15**) or NAADP (**14**) but in the absence of nicotinic acid the intramolecular attack of the adenine ring results in the cyclic product.

In addition to the Ca^{2+} mobilising properties of NAADP, cADPRP has also been shown to mobilise internal stores of Ca^{2+} in sea urchin egg homogenate and in rat brain microsomes.[95] This is in marked contrast with the reaction with NAD^+ where only the cyclic product is able to cause Ca^{2+} mobilisation and $NAAD^+$ has no such activity. The multifunctionality of these enzymes is novel and in addition, the fact that the products are intimately related to Ca^{2+} mobilisation suggests that this enzyme plays a pivotal role in Ca^{2+} signalling. What remains unclear is why the cell should link activation of these two distinct internal calcium stores so closely, and in which circumstances one system is used in preference to the other.

1.9 Synthesis of cADPR

Clearly, in order to improve our understanding of this system and elucidate the nature of the binding protein for cADPR, analogues of cADPR are required. The synthesis of such structurally modified compounds, each one with a small, yet hopefully significant, change from the parent compound, can often result in dramatic differences in biological activity.

Such compounds can be used as pharmacological tools for the investigation of biological properties and slowly a structure - activity profile can be developed. By careful evaluation of this information chemists and pharmacologists are able to generate an understanding of the important molecular motifs essential for activity and binding (pharmacophores). For example, non-hydrolysable analogues confer metabolic stability upon the molecule allowing its properties to be investigated in cells where the parent compound is rapidly hydrolysed, and fluorescent analogues can be used to label and identify the receptor. cADPR provides a wealth of opportunity to design such modified compounds.

A retrosynthetic analysis of cADPR indicates two initial possible disconnections which both result in a linear synthon: the breakage of the pyrophosphate bond to give a bisphosphate, or the breakage of the N1-ribosyl link to give a linear pyrophosphate (figure 1.9).

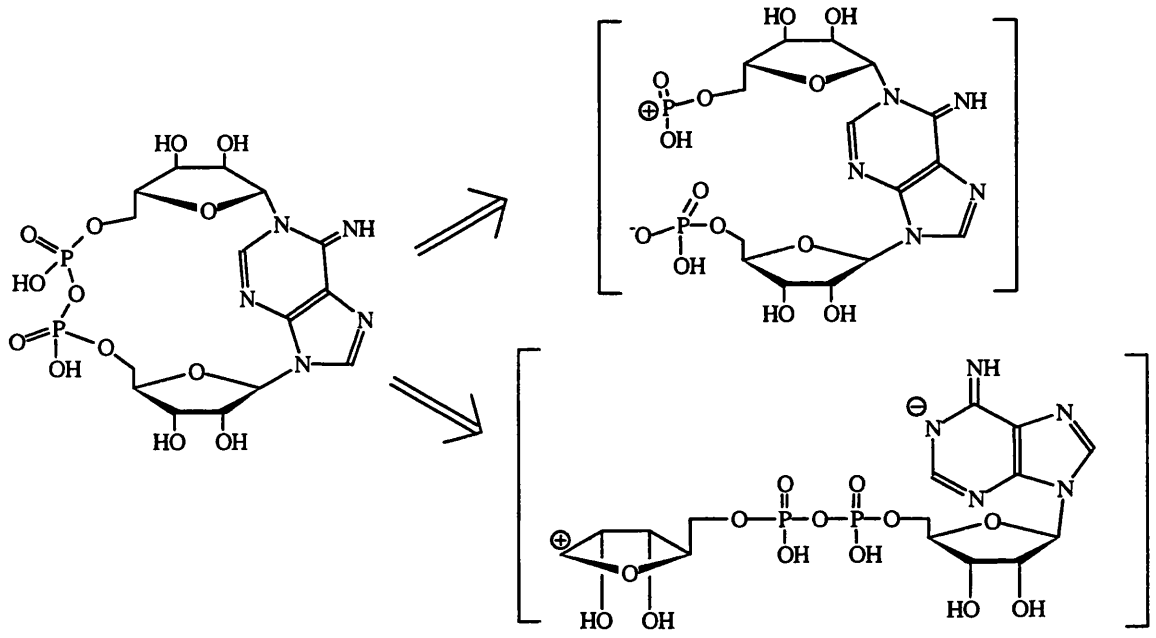


Figure 1.9. Retrosynthetic Analysis of cADPR.

From a chemical perspective the disconnection of the pyrophosphate is an essential first step since this moiety is particularly labile (section 4.1) and it is this bisphosphate which has been the target compound in this laboratory for a total chemical synthesis of cADPR and its analogues.[96] Although this approach has met with some success the cyclisation of the bisphosphate to form the cyclic dinucleotide has been problematic. Neither we, nor others, have yet been able to develop a useful total chemical synthesis of cADPR.

Breaking the N1-ribosyl linkage mimics the natural formation of cADPR since it is this bond which is formed by the enzyme. The forward step synthesis of this linkage requires the stereospecific nucleophilic attack of the N1 atom of the adenine ring at the ribose anomeric position with the displacement of a leaving group and the formation of the β-linkage. This stereoselective cyclisation is an ambitious chemical problem (section 5.1) and it was for this reason that we,[97] like others,[67] decided to perform this step enzymatically utilising *Aplysia* ADP-ribosyl cyclase. It was therefore necessary to present the enzyme with analogues which resembled its natural substrate and hence nicotinamide was the logical leaving group at the ribosyl anomeric centre. The chemical target compounds have now become modified analogues of β-NAD^+ (figure 1.10).

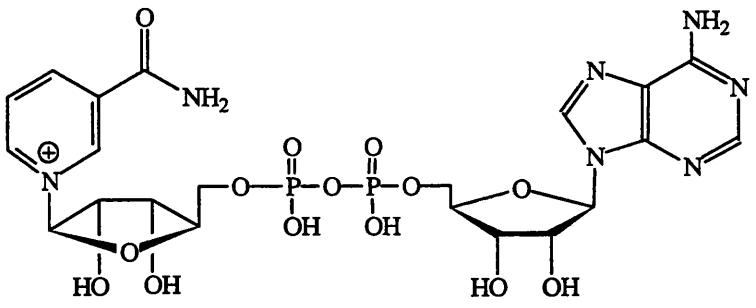


Figure 1.10 Structure of β-Nicotinamide Adenine Dinucleotide (β-NAD^+, **8**).

A similar retrosynthetic analysis of β-NAD^+ disconnected the pyrophosphate linkage to give two monophosphates, nicotinamide mononucleotide (NMN, **18**) and adenosine 5'-monophosphate (AMP, **19**), both shown (figure 1.11).

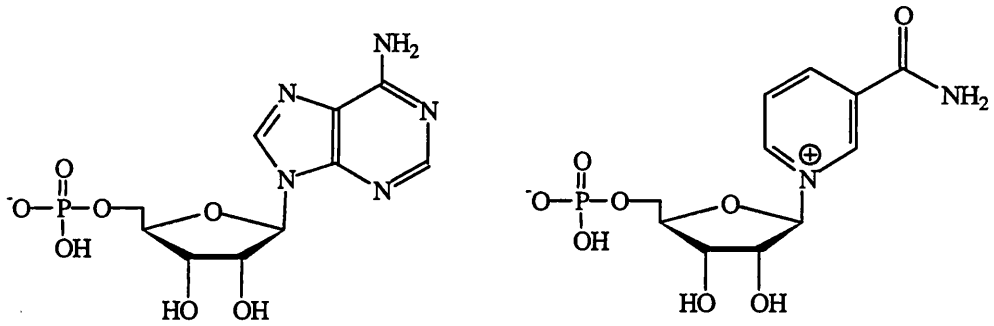


Figure 1.11 Structures of Both Adenosine 5'-Monophosphate (AMP, **19**) and Nicotinamide Mononucleotide (NMN, **18**).

Since the NMN half of the molecule is likely to be an essential motif for cyclisation, this monophosphate, which was commercially available, was kept constant and it was the AMP nucleotide which was altered. Further retrosynthesis of AMP disconnected the phosphate group to give the synthetic target nucleoside adenosine (**20**, figure 1.12).

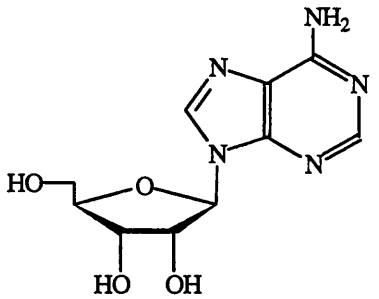


Figure 1.12 Structure of Adenosine (**20**).

In summary, the forward chemo-enzymatic synthesis of cADPR or its analogues involves the synthesis of analogues of adenosine, selective phosphorylation to give the resultant nucleotide, chemical coupling with NMN resulting in a β-NAD^+ analogue and enzymatic cyclisation to give the modified cADPR analogue. This route could readily allow for alterations in the purine ring, the adenosine ribose sugar or the pyrophosphate linkage with the only limiting factor being the substrate specificity of the cyclising enzyme. Fortunately, initial investigations have shown that ADP-ribosyl cyclase is able to accept a wide range of such modifications.[97]

Since each of these chemical precursors is in itself a biologically important molecule their properties and chemistry have been widely reviewed. Below is a brief introduction to some of the properties and biological importance of these intermediate parent compounds.

1.9.1 Adenosine (20) and Adenosine 5'-Monophophate (19)

Nucleic acids, the long thread like polymers which constitute the material of inheritance (DNA) and the means of translating this information (RNA) in nearly all living organisms, are made up of repeating units of monomers called nucleotides. These nucleotides, eg adenosine 5'-monophophate are the phosphoric esters of nucleosides, eg adenosine, and polymerise to form nucleic acids by the formation of a 3'-5' phosphodiester linkage.[98] The nucleotides themselves consist of three components:

(a) a nitrogen heterocyclic base of which there are two main types - monocyclic pyrimidines and bicyclic purines - adenosine is an example of a purine nucleoside,

(b) a pentose sugar which is either 2-deoxyribose in DNA or ribose in RNA,

(c) a phosphate residue.

Base - base recognition is an important feature in the correct functioning of the nucleic acid polymers and a key feature controlling this is the ability of the nucleotides to accept and donate hydrogen bonds. The acid base behaviour is therefore one of their most important physical features determining both charge and tautomeric structure. Figure 1.13 shows the states of protonation for AMP across the whole pH range.[98]

In addition to this extremely important role AMP, and a series of nucleotides related directly to it, have several other fundamental biological functions. AMP is readily converted in the cell to adenosine 5'-diphosphate (ADP, **21**), adenosine 5'-triphosphate (ATP, **22**) and cAMP (**1**). ATP is the primary source of free energy which plant, animal and bacterial organisms use for the performance of mechanical work, the active transport of molecules and ions, and the synthesis of biomolecules from their precursors.[99]

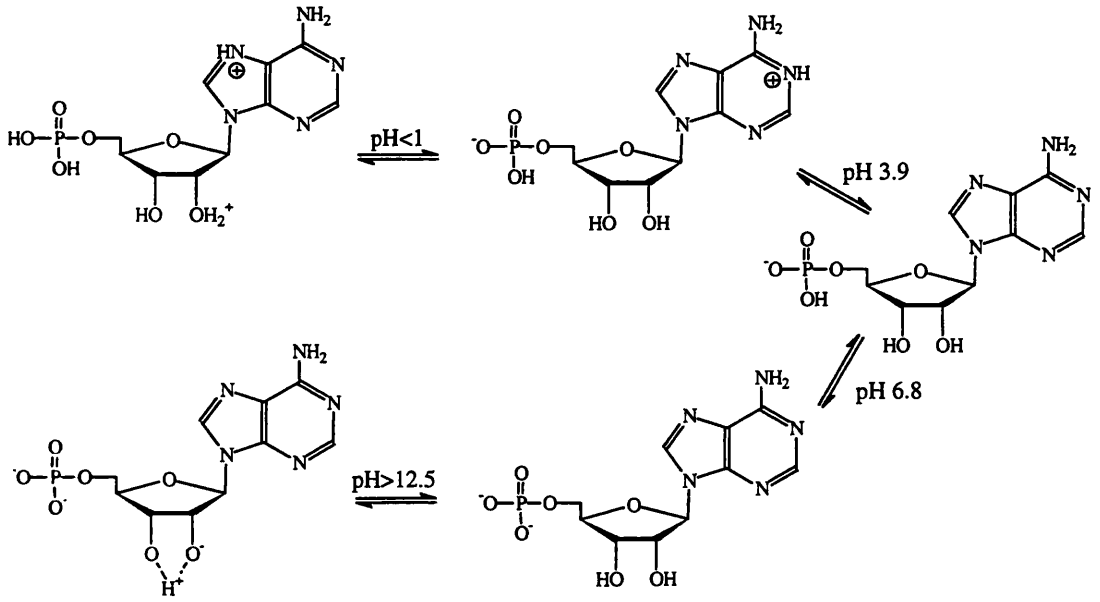


Figure 1.13 The States of Protonation of AMP (**19**) Across the pH Range.

ATP (**21**) is an energy rich molecule because its triphosphate ester contains two phosphoanhydride bonds. Hydrolysis of this linkage to give either AMP (**19**) or ADP (**21**) and inorganic pyrophosphate or monophosphate respectively has a considerable free energy. This energy is harnessed to drive reactions which require such an input, for example to initiate contraction in muscle fibres.[100] ATP is synthesised by organisms in two ways. Firstly the enzyme adenylate kinase is able to catalyse the interconversion of AMP, ADP and ATP so that a cell can maintain a steady equilibrium. Secondly ATP is formed by the phosphorylation of ADP, primarily in the cell's mitochondria, as a result of the oxidation of fuel molecules or photosynthesis. An excess of ATP is able to inhibit this pathway and conversely it is an excess of ADP which stimulates the mechanism.

A further diverse use of both ATP, adenosine itself, and in a more minor role ADP, is as neurotransmitters.[101] This role of ATP is quite distinct from the role discussed above and due to the universal role of ATP as an energy source, the notion that ATP was also a transmitter was resisted for a long time. ATP is contained in the synaptic vesicles of neurons and is released in a Ca^{2+} dependent fashion upon nerve stimulation. Exogenous ATP has been shown to mimic the effects of nerve stimulation [102] and ATP is also proposed to act as a conventional transmitter in the CNS and autonomic ganglia.[103] The ATP receptors respond to a variety of purine nucleosides but in general a better response is achieved with ATP than either ADP or AMP. One exception is in platelets where stimulation of the receptors, which respond selectively to ADP, causes platelet aggregation.

Adenosine itself produces many pharmacological effects (reviewed [104]) which are mediated by two distinct receptors linked to the inhibition or stimulation of cAMP respectively but are not sensitive to AMP, ADP or ATP.[105] There seems to be no special synthetic mechanism for the preparation of adenosine but it is produced by many tissues as a by-product of ATP breakdown. That there is no evidence for the storage of adenosine in vesicles or for its calcium dependent release leads to the conclusion that adenosine is probably not a true transmitter. However the pharmacological effects of adenosine are diverse and include vasodilatation of coronary vessels, inhibition of platelet aggregation, bronchoconstriction and inhibition of transmitter release at many synapses. For these reasons there is interest in the therapeutic potential of adenosine and its mimics for various conditions including hypertension, ischaemic heart disease and stroke.

The importance of cAMP as a second messenger has already been discussed (section 1.2).

1.9.2 Nicotinamide Adenine Dinucleotide (NAD^+, 8)

Aerobic organisms derive their free energy from oxidation of fuel molecules such as glucose and fatty acids. The lost electrons are not transferred directly from the fuel molecules to oxygen but are passed through a series of special carriers. It is then the reduced form of these carriers which transfer their high-potential electrons to O_2 by means of an electron transport chain located in the inner membrane of mitochondria. As a result of this flow of electrons, ATP is synthesised, and this is the major source of this important triphosphate in aerobic organisms.[100]

One of the major acceptors of electrons in this chain is NAD^+, less frequently referred to as diphosphopyridine nucleotide (DPN) or coenzyme I (CoI).[98] Following oxidation of a substrate, the nicotinamide ring of NAD^+ (**9**) accepts a hydrogen atom and two electrons, (figure 1.14) to produce NADH (**23**).[106] Thus, while AMP can be thought of as an energy carrier for many reactions, NAD^+ (**8**) can similarly be thought of as a hydrogen carrier.

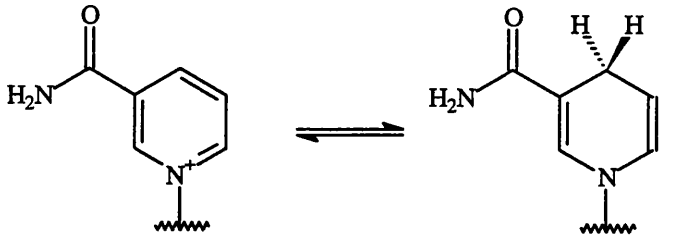


Figure 1.14 Reduction of the Nicotinamide Ring of NAD^+ (**8**) to NADH (**23**).

An example of a reaction for which NAD^+ acts as a coenzyme in this manner is the oxidation of ethanol to acetaldehyde and then further to acetic acid. The enzyme which catalyses this reaction is alcohol dehydrogenase, located mainly in the liver.

NADP (**12**), also known as triphosphopyridine dinucleotide (TPN) or coenzyme II (CoII) (section 1.8) is also a biological carrier of hydrogen with its reduced form being NADPH (**13**). This compound acts as a cofactor for a different class of reaction to NAD^+.[98] Whereas for the NAD^+ (**8**) - NADH (**23**) equilibrium NAD^+ acts as the electron acceptor, in the NADP (**12**) - NADPH (**13**) equilibrium it is NADPH which acts as the cofactor donating electrons in reductive biosyntheses, itself being oxidised to NADP. The electrons are carried by this compound in the same manner as in NAD^+ and the extra phosphate ester, at the adenosine ribose 2' position, acts as a tag directing the different

coenzymes to the correct enzymes. In the main these nucleotide dependent enzymes are highly specific for either NAD^+ or NADP.

Since NAD^+ and NADP are present in the cell only in small quantities, the coenzyme must be regenerated efficiently in order to prevent the glucose metabolism chain terminating. This is achieved by the pyridine nucleotides passing on their electrons to a subsequent carrier and as they do so the coenzyme is regenerated. However, despite the essential role that NAD^+ and NADP play in cell metabolism, animals are unable to synthesise nicotinamide and it is therefore an essential vitamin.[107]

1.10 Currently Known Structure Activity Relationships for cADPR

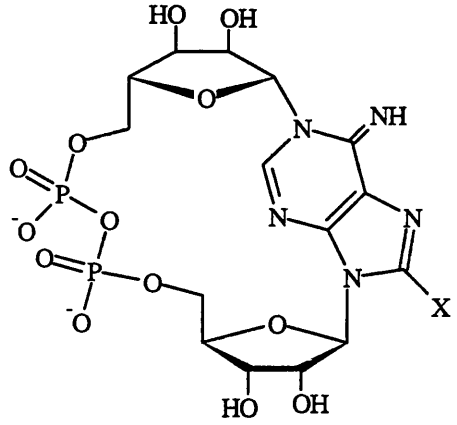
An understanding of the proteins which interact with cADPR is fundamental to the complete comprehension of the mechanism by which cADPR regulates Ca^{2+} release. This picture is no longer a simple agonist - receptor interaction but is complicated by the knowledge that calmodulin is required as an accessory protein to allow cADPR to stimulate release. In the absence of purification and characterisation of the cADPR binding proteins other methods have been employed to gain knowledge of this site.

Early work used conventional binding studies to investigate this site. The synthesis of [^{32}P]cADPR (**24**) by cyclisation of [^{32}P]NAD^+ (**25**), has allowed this radiolabelled compound to be used as a probe.[108] The radioactive material bound only to sites on the microsomes and could not be competitively displaced by NAD^+, ADPR, or $Ins(1,4,5)P_3$. However non-labelled cADPR was able to displace all the labelled material. These

studies provided further early evidence that cADPR was indeed operating through a different Ca^{2+} release mechanism to Ins(1,4,5)P_3.

The second approach to the characterisation of this binding site has been the use of photoaffinity labelling. [^{32}P]8-Azido-cADPR (**26**), a radiolabelled antagonist of the cADPR Ca^{2+} release mechanism synthesised from the NAD^+ analogue in the usual manner, was shown to act at the same binding site as cADPR by competitive displacement of the antagonist by the parent compound.[109] After binding of [^{32}P]8-azido-cADPR, the complex was photolyzed using UV light to give a fluorescent product. This identified two labelled proteins to which the analogue had bound, one of 140kDa and the other of 100kDa, both of which are far smaller than the known subunits of the ryanodine receptors. Furthermore these proteins were specific for cADPR and this suggested that cADPR may not act directly at the ryanodine receptor. This may explain why antagonists of the cADPR Ca^{2+} release do not block the Ca^{2+} releasing actions of caffeine or ryanodine.[67]

The third method of characterising the binding protein has been by the synthesis of analogues of cADPR and the characterisation of their pharmacological properties. This field has been unusual in that the first analogues synthesised were in fact antagonists of the Ca^{2+} release mechanism. Walseth and Lee [67] synthesised a series of compounds which were substituted at the 8-position with a bromine atom (**27**), an azido group (**28**) or an amino group (**11**) (figure 1.15).



X=Br (**27**) 8-Br-cADPR
X=N_3 (**28**) 8-azido-cADPR
X=NH_2 (**11**) 8-amino-cADPR

Figure 1.15 Structures of 8-Substituted Analogues of cADPR.

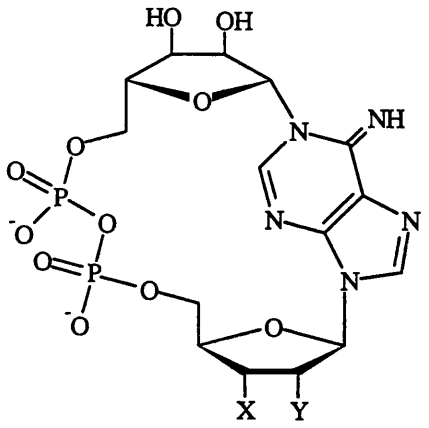
Pharmacological studies using these compounds indicated the 8-position to be of critical importance to Ca^{2+} release since none of these analogues was able to mobilise any Ca^{2+}. Additionally these compounds blocked cADPR-stimulated Ca^{2+} release with 8-amino-cADPR (**11**) being the most potent. These effects were shown to be competitive with the inhibition being overcome by high concentrations of cADPR, and competitive binding studies have confirmed that all the compounds act at the same binding site. The first implication of these results is that occupation of the receptor site does not automatically result in Ca^{2+} release.

These antagonists have been instrumental in furthering the investigation into both the mechanism and physiological role of cADPR induced Ca^{2+} release. In a similar manner to that used to investigate the role of Ins(1,4,5)P_3 using its antagonist heparin, the cell is exposed to one of these antagonists. By monitoring the subsequent cellular functioning it is possible to determine which functions have been affected by the loss of cADPR action. These methods have been used particularly in sea urchin eggs to study individually the multiple Ca^{2+} transients which occur at fertilisation.[110] Other use of these antagonists has

confirmed the presence of cADPR induced calcium release in different tissues.[70, 84] The limitation of the currently known antagonists is that, due to their chemical instability, they are unsuitable for microinjection work and therefore they can not be used to investigate transients in intact cells. Similarly, their effects in microsomes are short lived, therefore an antagonist with increased resistance to both chemical and enzymatic hydrolysis would be a powerful pharmacological tool. Other substitutions at the 8-position have been made to try to further investigate this effect, namely the 8-$NHCH_3$ (**29**), 8-$N(CH_3)_2$ (**30**), 8-OH (**31**), 8-OCH_3 (**32**) and the 8-piperidino (**33**) derivatives.[97] All of these compounds were antagonists but none was more powerful than 8-amino-cADPR (**11**) compound already known and a study of molecular volume of the substituents has suggested that as the molecular volume at this position increases the degree of antagonism decreases.[111] For the 8-OH analogue it is of course not molecular volume that plays a role but this may be a reflection of the H-bonding requirements for antagonism since this analogue exists mainly in the carbonyl-, and not the hydroxy- form.

A second area of potential interest for the production of cADPR analogues which probe the cADPR binding protein interaction is the adenosine ribose ring. As previously discussed (section 1.8) cADPRP (**17**) is able to induce Ca^{2+} release in mammalian tissue in a similar manner to that of cADPR and was more potent.[95] This indicates that the 2' hydroxyl is not important for activity, and the synthesis and testing of 2'-deoxy-cADPR (**34**), which had the same Ca^{2+} mobilising properties as cADPR itself, adds further evidence to this supposition.[112] The importance of the 3' hydroxy group has also been investigated by the synthesis and testing of 3'-deoxy-cADPR (**35**) and 3'-cADPRP (**36**). It has been reported that 3'-cADPRP does not elicit any Ca^{2+} mobilising activity [113] and

biological testing has demonstrated that 3'-deoxy-cADPR is only a very poor agonist of this release mechanism.[112] Binding data for 3'-deoxy-cADPR showed that it was able to competitively displace labelled cADPR and it therefore appears that the 3'OH group is important only for the activation of Ca^{2+} release, and not for recognition or binding. This area of cADPR obviously warrants further investigation.



cADPR (**7**) X=Y=OH
cADPRP (**17**) X=OH, Y=OPO_3^{2-}
3'-cADPRP (**36**) X=OPO_3^{2-} , Y=OH
2'-deoxy-cADPR (**34**) X=OH, Y=H
3'-deoxy-cADPR (**35**) X=H, Y=OH

Figure 1.16 Structures of 2' and 3' Modified cADPR Analogues.

Of enormous use to pharmacologists are the so called "caged" analogues. These are compounds which are protected with photoactive hydrophobic groups to render them inactive and then once inside the cell the protecting groups are removed by photolysis to yield the active metabolite. Caged cADPR (**37,** not shown) has been synthesised with either one or other phosphate protected with a (2-nitrophenyl)ethyl group.[114] A mixture of these two regioisomers could be separated by HPLC and the protecting group removed by exposure to 350nm UV light for 2h. The caged forms of cADPR were not themselves able to induce any Ca^{2+} release but the uncaged compound did elicit Ca^{2+} release. That this release was antagonised by the addition of 8-amino-cADPR confirmed the release to be a result of cADPR agonism. The use of these compounds allows a number of

problems associated with calcium leakage with microinjection and Ca^{2+} contamination of samples to be overcome.

As a result of the topical nature of this work and the necessity to rely upon a chemo-enzymatic synthetic route, few other chemically modified analogues of cADPR have been reported. Zhang and Sih have reported the synthesis of two purine modified amino-bridged compounds in their search for active stable fluorescent probes for investigation of the binding protein (figure 1.17).[115-116] The pseudo N7 cyclised compound (**38**) was formed using enzymatic cyclisation and the compound cyclised at the pseudo N6 amino (**39**) group was formed in low yield by a chemical synthetic route (section 5.2.2). When cyclised at the pseudo N6 amino position (**39**) the analogue was able to release Ca^{2+} from rat brain microsomes and was approximately twice as potent as cADPR in this preparation. The other regioisomer was unable to elicit Ca^{2+} mobilisation.

The enzyme catalysed cyclisation of nicotinamide adenine triphosphate (**40**) has also been reported to give cyclic adenosine 5'-triphosphate ribose (**41**) which was a more potent Ca^{2+} mobilising agent than cADPR when measured in rat brain microsomes.[117]

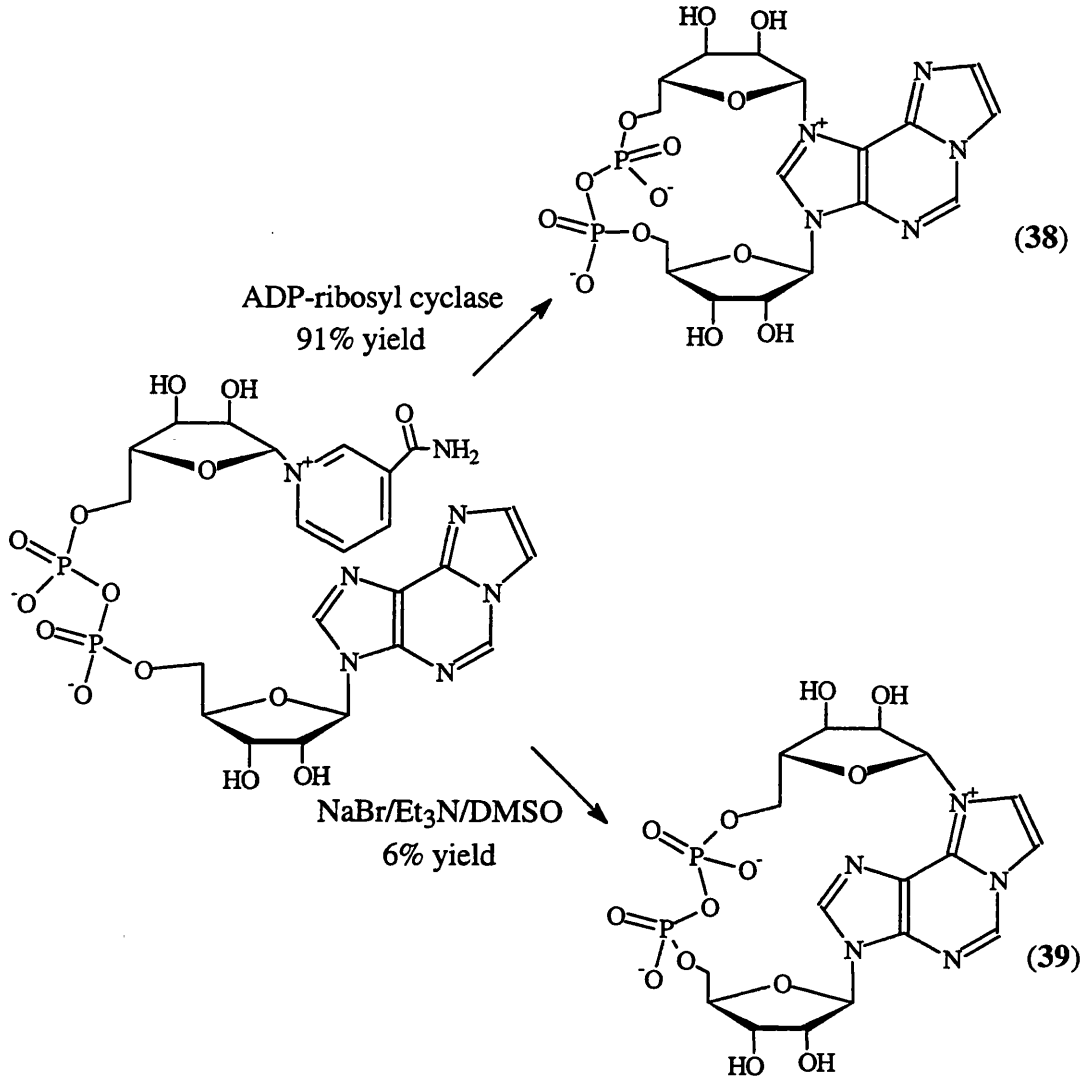


Figure 1.17 Synthesis of Etheno Bridged Analogues of cADPR.

Figure 1.18 is an hypothetical diagram which summarises the known structure activity relationships which exist between cADPR and its binding protein.

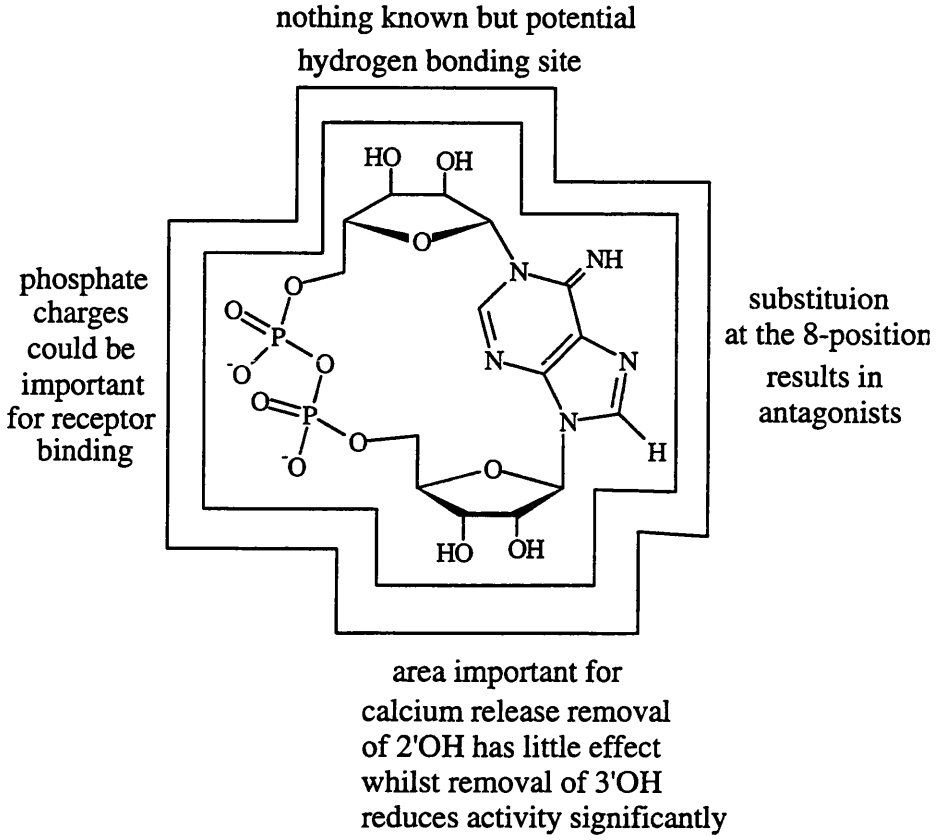


Figure 1.18 Diagram to Show Known cADPR-Binding Protein Structure Activity Relationships.

The use of a chemo-enzymatic approach in the synthesis of cADPR analogues allows for the investigation of the structural requirements of the cyclising enzyme for the recognition of the substrate and formation of the product, and this will be discussed further (chapter 5).

1.11 Aims of this Project

To facilitate thorough investigation of the biochemical properties of a metabolic pathway the investigator requires compounds which are metabolically stable, antagonists, fluorescent and membrane permeable. The medicinal chemist, as well as having these

objectives in mind, is interested in rapidly ascertaining any pharmacophores so that small molecule mimics can be synthesised with the eventual aim of providing lead compounds for therapeutic studies. It was with these general objectives in mind, to investigate the limits of the cyclising enzyme and to build upon the known structure-activity relationships that the target compounds of this thesis were designed. Four particular areas of the parent compound became the targets for modification.

(a) To further investigate the requirements of the adenosine ribose ring hydroxyls for Ca^{2+} mobilisation three compounds were designed (i) in which the stereochemistry of the 2'OH was reversed, (ii) in which the 2', 3' diol was protected with an isopropylidene group and (iii) in which the ribose ring was replaced with a flexible methoxy ethoxy chain.

(b) To investigate the role of the adenosine furanoside oxygen an analogue was designed in which the ribose ring was replaced with a suitable cyclopentyl ring.

(c) To further investigate the role of the purine ring on antagonism the effect of the N7 atom was investigated. The initial target analogue had this nitrogen replaced by a methine group and then the effect of this alteration combined with substitution at the 8-position was also investigated.

(d) To investigate the structural requirement of the N6 amino group by cyclisation of the inosine, guanosine and the deaminated purine analogues.

In a more general sense, the aim of the project was to develop, and use, a widely applicable synthetic route which could be used to convert any analogue of adenosine into an analogue of cADPR with the minimal use of protecting groups and within the limitations of the cyclising enzyme. The rest of this thesis describes the methods by which these aims were achieved. The biological results which were obtained from the

testing of the cyclic analogues by either ourselves or our collaborators at the University of Oxford are also reported.

CHAPTER TWO

SYNTHESIS OF MODIFIED NUCLEOSIDES

2.1 Introduction

The first step in the chemo-enzymatic route to analogues of cADPR (section 1.9) with structural diversity in the adenosine moiety was to synthesise a series of adenosine like compounds with the desired chemical modifications. This chapter, the first of four chemical discussion chapters, describes the synthesis of these modified adenosines. Those compounds synthesised can broadly be divided into two categories, those altered in the ribose ring and those altered in the purine ring, and were determined by the overall aims of the project (section 1.11).

Following developments in the understanding of the biological significance of nucleosides, which became apparent in the early 1950's, the synthesis of such compounds has become instrumental in identifying not only natural nucleosides and their metabolites, but also the effects of synthetic nucleosides on living systems. From the first historic synthesis of such compounds by Emil Fischer in 1914, synthetic analogues have now been developed with both potent antibiotic [118] and antiviral [119] activity. A host of modified purine nucleosides is now known with modifications involving all positions of the parent molecule.

The chemistry of purine nucleosides is centred around the two distinct moieties - the carbohydrate ribofuranoside and the aromatic heterocyclic ring. In principle the nucleoside as a whole is able to successfully react in the manner expected of either moiety individually, but in practice, careful protection may be required to minimise side

reactions. The excellent work by L. B. Townsend [120] comprehensively summarises the many different possible reactions.

An important area of focus of the nucleosides is the glycosidic bond which links the ribose ring to the purine base, since this can undergo hydrolysis with important chemical and biological implications. Models have been suggested for both acid and base catalysed cleavage, although the acidic hydrolysis has been more thoroughly investigated.[121]

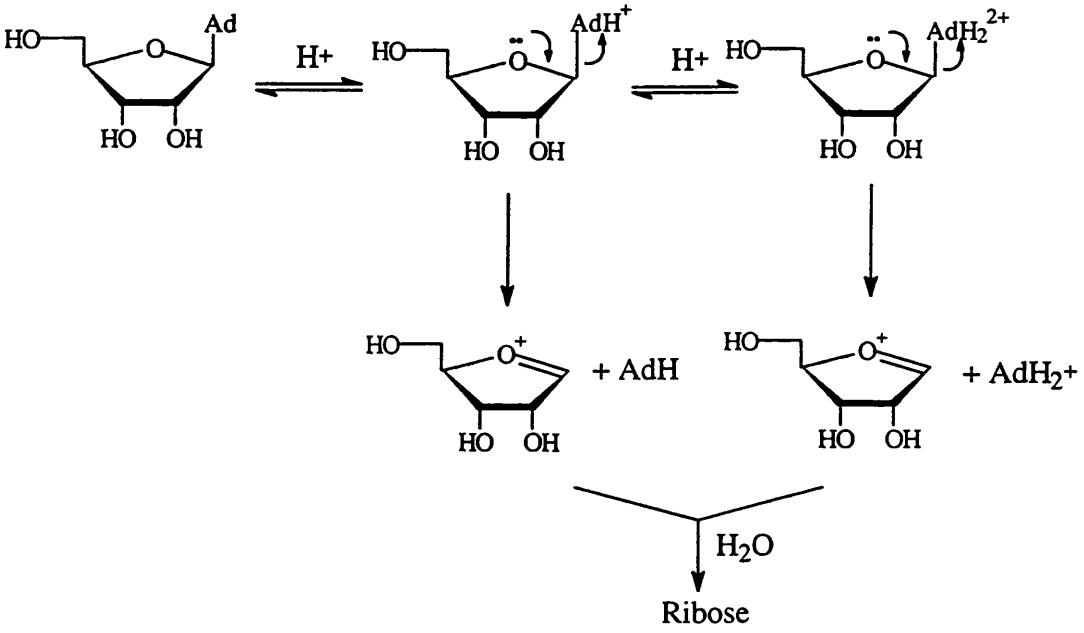


Figure 2.1 Proposed Mechanism for Acidic Hydrolysis of Purine Nucleosides.

The mechanism (figure 2.1) proceeds via protonation of the purine base, followed by cleavage of the glycosidic bond, to yield the ribose and purine moieties individually. In general, purine nucleosides are stable within a moderate pH range at room temperature, but the rate of hydrolysis is, of course, affected by substituents on either the carbohydrate

ring or the purine ring. For example, removal of electron withdrawing groups from the ribose ring stabilises the glycosyl carbonium ion enhancing the possibility of hydrolysis, so that 2′-deoxy compounds are more susceptible to hydrolysis than the corresponding ribonucleoside.[121]Similarly, a decrease in the electron density at the base - ribose linking nitrogen by substitution of the ring with electron-withdrawing groups, facilitates deglycosylation.[122]

2.2 Synthesis of 2′, 3′-*O*-Isopropylidene Adenosine (42)

Formation of cyclic isopropylidene acetals is a standard method for the protection of diols. However, one of the problems associated with the protection of *cis* diols of purine nucleosides with this group is the formation of insoluble salts between the purine base and the acid catalyst in the reaction. As a result there are many different protocols published to afford this protection and the results can be very variable.

The traditional approach to the synthesis of isopropylidene acetals is the reaction of a diol with dry acetone in the presence of an acid catalyst and the most commonly used acid for this type of reaction is *p*-toluene sulphonic acid (TsOH).[123] However, as a molecule of water is generated in the reaction, some means of dehydration is usually required to prevent the acetal being rapidly cleaved again soon after formation. Azeotropic removal of water is not feasible due to the low boiling point of acetone so a drying agent such as molecular sieves or CuSO4 is required.[124]

An alternative method to the formation of 2′, 3′-*O*-isopropylidene derivatives is to use acetal exchange with 2,2-dimethoxypropane.[125] Since this reaction liberates two moles of MeOH there is no need for a dehydrating agent, and when the reaction is thermodynamically favoured the exchange will go to completion. There was literature precedent for this method being used for the synthesis of 2′, 3′-*O*-isopropylidene adenosine (41) and so this method was the first to be attempted. A large excess of TsOH was stirred with a large excess of acetone and to this was added dimethoxypropane and adenosine.[126] Yields from this reaction were very variable (0-49%) depending largely upon the quality of the acetone and the percentage of dehydration that was achieved of the reagents before reaction.

In addition, the reaction was further complicated by the formation of acetone-insoluble salts, and it was for these reasons that a second method was sought.

The second method used triethyl orthoformate both as a drying agent, and as an activating agent, reacting with the acetone forming a 2,2-diethoxy propane intermediate (figure 2.2).

Triethyl orthoformate was added dropwise to a suspension of adenosine and TsOH in acetone and the mixture was stirred until a clear solution was seen.[127] The reaction was neutralised by addition to dilute ammonium hydroxide and, after removal of the solvent *in vacuo*, the product (**42**) was purified by recrystallisation from ethanol. Although the reagents still had to be vigorously dried before use, this method seemed to provide more reliable results, with a consistent yield of 45%. It was important to remove any residual

traces of TsOH from the product to prevent the acid catalysed cleavage of the isopropylidene group in the subsequent phosphorylation step.

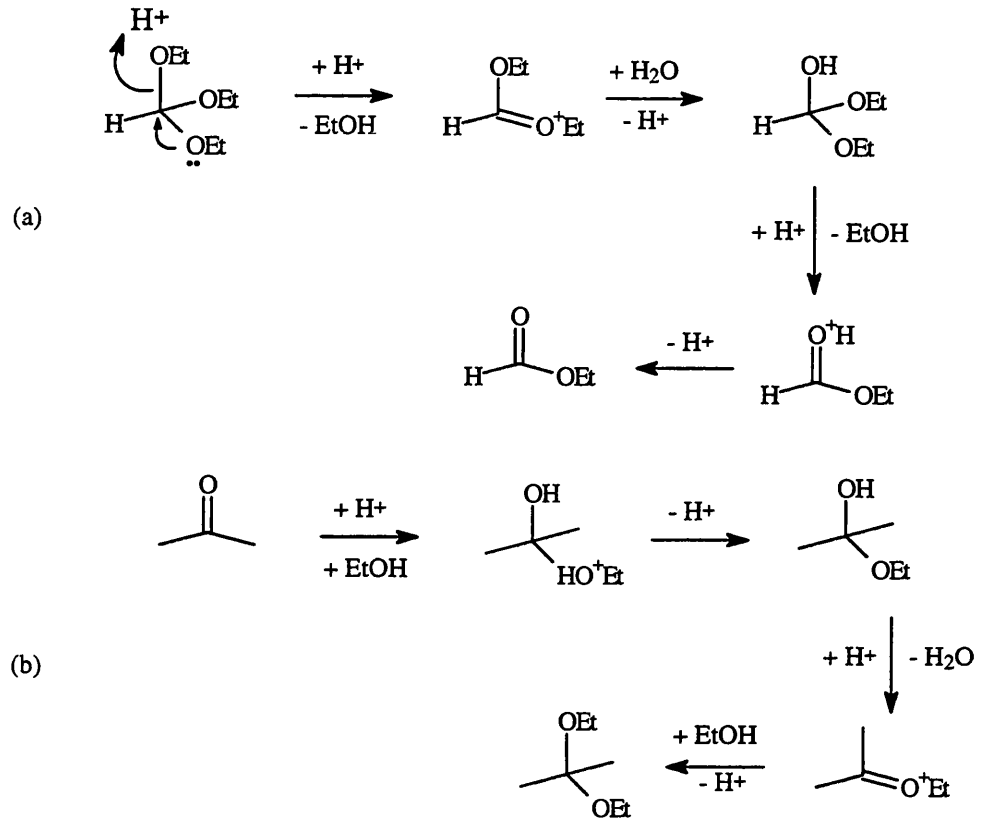


Figure 2.2 Mechanism of (a) Triethyl Orthoformate as a Drying Agent and (b) Activation of Acetone.

The formation of such isopropylidene acetals is thermodynamically driven and, in the case where two acetal products are possible, the more stable isomer will prevail. Where more than 1 product is possible the more stable isomer is dependent upon the carbonyl being used and it is well established that in general ketones tend to result in five membered 1,3-dioxolane ring products and aldehydes favour six membered 1,3-dioxane rings.[128]

Although adenosine has three hydroxy groups that may react with acetone, the formation of the 3′, 5′-*O*-isopropylidene acetal would never be possible due to the large steric constraint imposed by the *trans* orientation of the 3′ and 5′ groups about the ring. However, the 5′OH is the most reactive hydroxyl site, and an hemiacetal intermediate probably forms here initially. Since this is unable to react further, to give an acetal, it would be rapidly cleaved followed by further reaction to give 2′, 3′-*O*-isopropylidene adenosine (**42**) as the only regioisomer. To corroborate this discussion a section of the ^1H NMR spectrum of the product is shown (figure 2.3). The 5′-OH group appears as a triplet indicating that it is directly bonded to a CH2 group confirming the acetal is formed between the 2′ and 3′ hydroxyls.

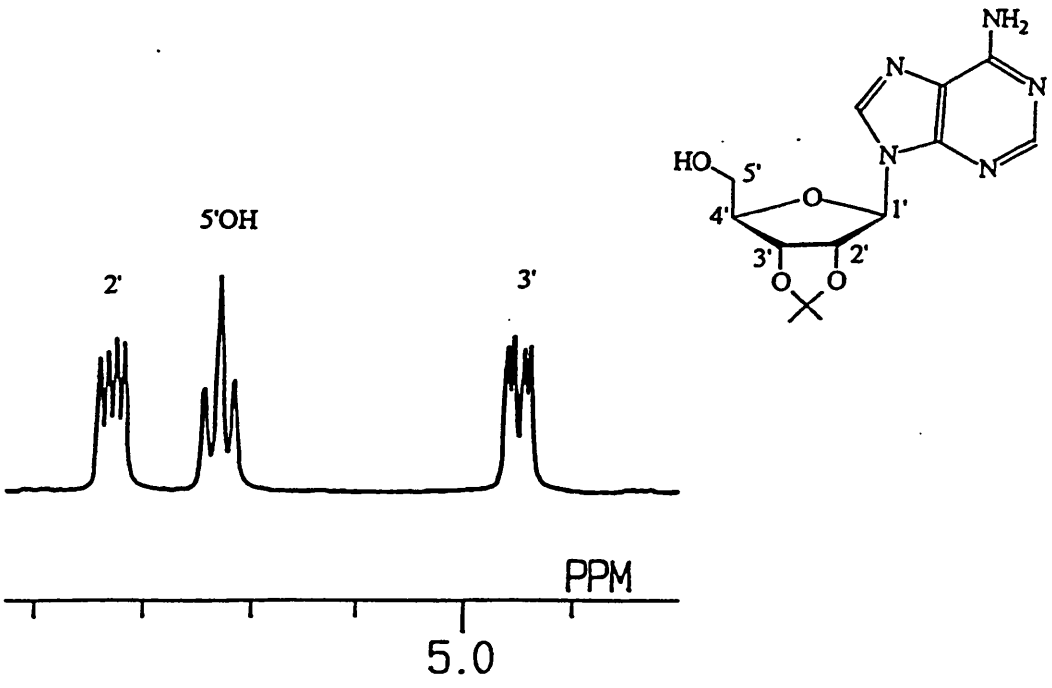


Figure 2.3 Part of the ^1H NMR Spectrum of 2′, 3′-*O*-Isopropylidene Adenosine (**42**) Showing the 5′OH Group (400MHz, solvent d_6DMSO)

This steric constraint is further illustrated by the addition of an isopropylidene group to xylose (**43**) (figure 2.4). In this case the 2′ and 3′ substituents are now *trans* with respect to each other, and although the preferred size of the resultant acetal would be a 1,3-dioxolane five membered, ring the steric constraint is such that 3′, 5′-*O*-isopropylidene xylose (**44**) is the only product formed.[129]

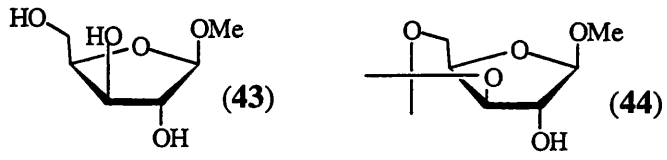


Figure 2.4 Structure of Xylose (**43**) and 3′, 5′-*O*-Isopropylidene Xylose (**44**).

2.3 Synthesis of 2′, 3′-*O*-Benzylidene Adenosine (45)

When reviewing the literature for the preparation of 2′, 3′-*O*-arylidene protected adenosine like compounds there were several examples in which the 2′, 3′-*O*-*p*-methoxy bezylidene adenosine (**46**) had been reported [130-131] but, in contrast, the preparation of 2′, 3′-*O*-benzylidene adenosine had not been fully discussed. In 1949 Michelson and Todd [132] published the synthesis of what they described as 3′, 5′-*O*-benzylidene adenosine. Given the initial generalisation (section 2.2) that aldehydes tend to result in acetal products containing 1,3-dioxane rings, this might be the expected product. However, as also already explained, this product is not possible due to the steric constraints imposed by the relative stereochemistry of the hydroxyls about the ribose ring. It was thus assumed that Michelson and Todd had in fact synthesised the 2′, 3′-*O*-benzylidene

adenosine (**45**) but, since this work had been carried out before the advent of NMR, they had incorrectly assigned the product. The work of Bakthavachalam *et al.* [133] did indeed confirm this since they reported the synthesis of 2′, 3′-*O*-benzylidene adenosine (**45**) but referenced the Michelson and Todd paper when describing the methodology. Surprisingly however, they made no mention of the fact that Michelson and Todd had reported the incorrect compound and, despite publishing extensive ^{1}H NMR data, they did not fully report other spectroscopic analysis of the compound. As a result, although this compound was first synthesised in 1949, it still remains largely uncharacterised in the literature to the best of the author's knowledge.

The preparation of 2′, 3′-*O*-benzylidene adenosine (**45**) adopted by Michelson and Todd in 1949 used the traditional method of forming an acetal - namely adenosine, benzaldehyde and ZnCl2 as the Lewis acid catalyst. However, the work up for the reaction was very complicated and so initially newer methodologies for the synthesis of this compound were explored.

The first method attempted was the reaction of the diol with benzaldehyde dimethyl acetal (acetal exchange) in the presence of TsOH acidic catalysis, the method of Horton and Weckerle.[134] This was known to produce the 2′, 3′-*O*-benzylidene protected product (**47**) when reacted with methyl-β-D-ribofuranoside (**48**).[135] Adenosine was stirred with benzaldehyde dimethyl acetal, TsOH and DMF in a flask fitted with an air condenser which was attached via a three way tap to a water pump. The flask was evacuated and heated to 50°C with the hope that methanol would be evaporated as soon as it was

formed, thus driving the reaction to completion. However this reaction gave little or no discernible product by TLC and after several attempts a new method was sought.

In a second attempt to avoid the prolonged work up of Michelson and Todd, the method which had been used to successfully synthesise 2′, 3′-*O*-isopropylidene adenosine (**42**) was adapted to try to synthesise the benzylidene adduct. Dry adenosine was added to a stirred solution of TsOH in freshly distilled and dried benzaldehyde followed by triethyl orthoformate dropwise. For the isopropylidene adduct an indication that the reaction had proceeded was that the mixture was seen to give a clear solution after 1-2h. However, even with prolonged stirring and gentle heating, a clear solution could not be obtained in the benzylidene case and again this method still gave no discernible product.

2′, 3′-*O*-Benzylidene adenosine (**45**) was finally synthesised using essentially the methodology of Michelson and Todd.[132] Since it has been shown that the formation of the benzaldehyde-zinc chloride complex, which is a key intermediate in promoting high yields of the benzylidene acetal, does not proceed as readily if the sugar is first added to the reaction mix [136] then a slight alteration was made to this methodology. Thus, instead of introducing all the reactants together, the ZnCl2 was initially stirred with the benzaldehyde until a milky gelatinous paste formed, approximately 30min, and adenosine was then added in one portion and the mixture cleared to give a solution, approximately 1h. After stirring overnight the 2′, 3′-*O*-benzylidene adenosine was purified by the complicated work up reported by Michelson and Todd. The product was first precipitated as its zinc chloride salt, washed with ether and dissolved in ethoxy ethanol. Addition of aqueous NaOH and making the pH more alkaline using CO2 gas caused the subsequent

precipitation of any inorganic salts. Filtration to remove these impurities followed by removal of the solvent and recrystallisation led to the product in 35% yield. The work up, as feared, was not easy to perform and substantial product was thus lost in the procedure leading to a yield approximately half of that reported. However, it is certain that this could have been improved if the reaction had been repeated. In a similar manner to that described for 2′, 3′-*O*-isopropylidene adenosine (**42**) the regioselectivity of the product (**45**) was confirmed by the 5′OH appearing as a triplet in the ^{1}H NMR spectrum (figure 2.5).

The synthesis of 2′, 3′-*O*-acetal adenosines from aldehydes or unsymetrical ketones leads to the formation of two possible diastereoisomers - an *endo* and *exo* compound (figure 2.6) being the kinetic and thermodynamic product respectively.

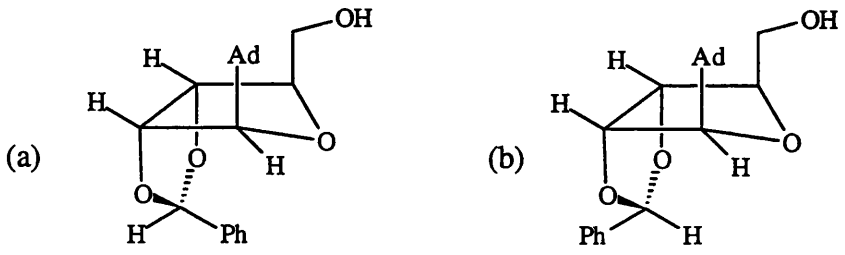
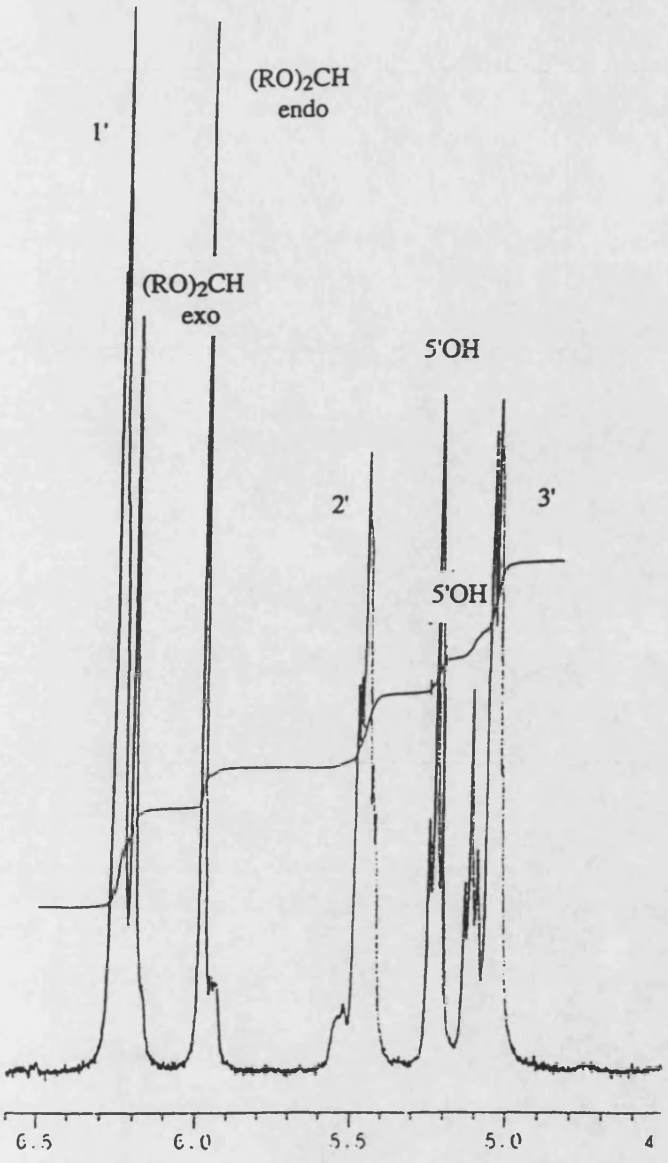


Figure 2.6 Schematic Representation of (a) *Endo* and (b) *Exo* 2′,3′-*O*-Benzylidene Adenosine (**45**).

Interestingly Bakthavachalam *et al.*,[133] who synthesised a whole series of adenosine acetals, were only able to isolate both stereoisomers in two cases, one of which was the benzylidene compound.



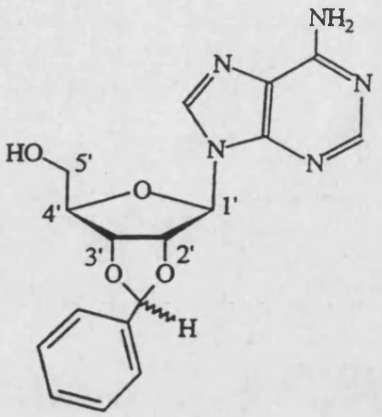


Figure 2.5 Part of the ^{1}H NMR Spectrum of 2′, 3′-*O*-Benzylidene Adenosine (**45**) Showing the 5′OH Group and the *endo* and *exo* Signals (270MHz, d6DMSO).

By using NOE NMR spectroscopy irradiating at the H4′ frequency and looking for an enhancement of the acetal H signal they were able to unambiguously assign the two

stereoisomers. The NMR data as reported was then used to assign the signals for each of the two diastereoisomers in the product mixture revealing a 1:1 ratio of the *endo*:*exo*, or kinetic:thermodynamic product from this reaction method (figure 2.5).

2.4 Synthesis of 9-(2-[Hydroxyethoxy]Methyl) Adenine (49)

The advent of Acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine (**50**), as a potent antiviral agent with activity against the Herpes simplex virus,[137] led, in the 1970's and 1980's, to the search for a facile synthesis of functionalised 2-hydroxyethoxymethyl derivatives of purines and pyrimidines. Initial work in the purine area led to several published routes, among them alkylation of 6-chloropurine (**51**) with (2-benzyloxyethoxy) methyl chloride (**52**),[138] and (2-trimethylsilyloxyethoxy)methyl iodide (**53**).[139] These reports quoted alkylation yields of 44% and 72% respectively and, following ammonolyisis and deprotection, 9-[(2-hydroxyethoxy)methyl] adenine (**49**) was obtained.

A third route to this class of compound, the most direct method to 9-[(2-hydroxyethoxy)methyl] adenine (**49**), was published by Robins and Hatfield in 1982.[140] They had developed alkylating agents and methodolgy such that a series of heterocycles, including adenine itself, could be directly alkylated thus providing a method for the synthesis of the title compound which combined direct alkylation of adenine with the minimum need for protection (figure 2.7).

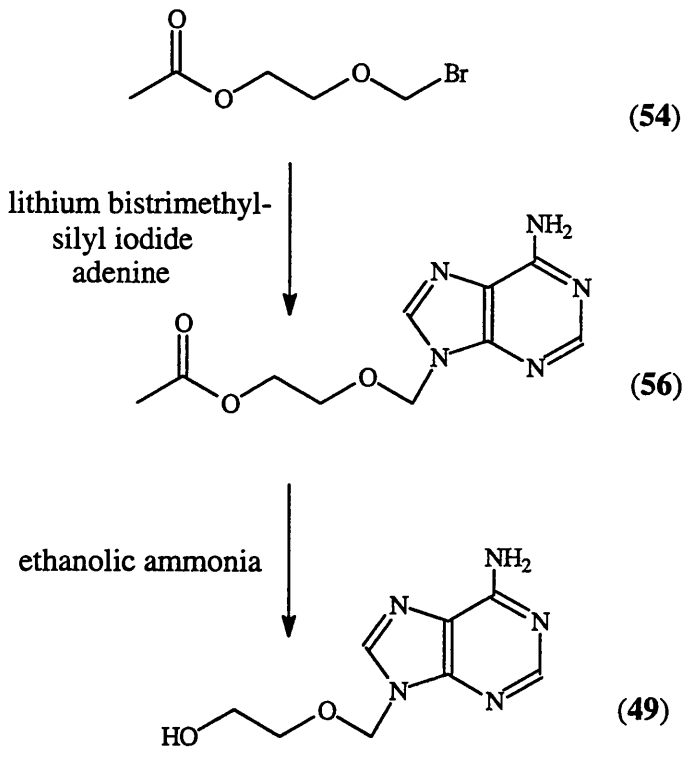


Figure 2.7 Synthetic Route to 9-[(2-Hydroxyethoxy)Methyl] Adenine (**49**).

The alkylating agent (2-acetoxyethoxy)methyl bromide (**54**) was readily prepared by the dropwise addition of 1,3-dioxolane to cooled freshly distilled acetyl bromide.[141] Vacuum distillation gave a clear liquid product with a boiling point of 85°C at 3mmHg (58-60°C 0.1mmHg [140]). This reagent was used within one month of its preparation since it was seen to degrade with time.

Alkylation of adenine (**55**) proceeded via deprotonation of the N9 position resulting in formation of an anion. Dry adenine was stirred in a large volume of pure dry DMF (1.1mmol in 25ml) and to this was added NaH as a suspension in mineral oil (1.2mmol). When deprotonation was complete, determined by no further observed H2 release, the

reaction mixture was cooled to -63°C. Subsequent reaction of the alkylating agent with this anion led to no discernible product despite thorough drying of the reagents and solvents (DMF was dried by distillation from BaO). ^{1}H NMR indicated intramolecular cyclisation of the alkylating agent, a process which would be favoured by Baldwin's rules.[142] It was thought that this had occurred due to incomplete formation of the adenine salt preventing rapid alkylation or due to reaction of excess hydride ions with the alkylating agent.

In order to try to improve the formation of the adenine anion before use the NaH was washed with dry hexane, under an atmosphere of nitrogen, to remove the mineral oil. This material, which was now very susceptible to rapid reaction with moisture, was suspended in dry DMF and added to a solution of adenine in DMF using a rapid flow of nitrogen through a double tipped needle. Stirring the solution at room temperature now afforded a clear solution which was cooled to -63°C. However, upon cooling, some of the adenine salt was seen to precipitate but, after reaction with the alkylating agent, some product (**56**) was isolated. The low yield, a disappointing 20% (75% [140]), was thought to result from the precipitation of the adenine salt. Despite attempts to increase this yield by addition of the alkylating agent over time, reaction at room temperature and dilution of the reaction mixture, no improvement could be made. It is of interest to note that Hendry *et al.* [143] also report very low yields, 14%, when trying to repeat this procedure.

Lithium bis(trimethylsilyl) amide is a sterically hindered non-nucleophilic base[144] and it was the use of this reagent to deprotonate adenine which did provide an improvement to this alkylation reaction. The lithium salt of adenine was more soluble in DMF than the

sodium salt and, as a result, the adenine anion remained in solution even when cooled to -63°C. Upon addition of the alkylating agent the reaction now proceeded but, although the yield was still low, it was consistently 5-10% better than before.

The final step in this synthesis was to deprotect the alkylated product (**56**) to yield the title compound (**49**). The method of Robins and Hatfield [140] was attempted first using sodium methoxide at room temperature. Although TLC showed complete deprotection following work up, using Amberlite IR-120 (H^+) resin, no discernible product could be isolated and unfortunately, the product had almost certainly been irretrievably bound to the resin.

The acetate group was successfully removed using ethanolic ammonia at 50°C. Removal of the solvent, followed by recrystallisation from MeOH, gave the product in 85% yield. It is of interest to note that deprotection of the acetate to give a hydroxyl group places both the CH2 groups of the ethyl chain into a similar electronic environment and they now appear as one peak in the ^1H NMR spectrum instead of two AB systems as in the protected compound (figure 2.8).

2.5 Synthesis of 7-Deaza-8-Bromo-Adenosine (57)

The introduction of a bromine atom into the 8-position of the heterocyclic ring of various purine nucleosides has provided intermediates that have proved invaluable for the preparation of many 8-substituted derivatives.[145] Mild reagents are required for this electrophilic substitution in order to avoid glycosidic cleavage and the standard procedure is to use aqueous conditions. Thus the 8-bromo derivative of adenosine, and similarly

AMP, ADP, ATP and cyclic AMP, have been obtained by reaction with bromine in a pH4 sodium acetate buffer at room temperature.[146]

The analogous bromination of 7-deazaadenosine (**58**, Tubercidin) provides a number of problems. Firstly there are two possible sites of bromination, both the 7 and 8 positions, and secondly neither position is reactive enough to prepare the product using bromine water. However, due to the unique activity of 7-bromo-7-deazaadenosine (**59**) as a reversible inhibitor of heterogeneneous nuclear RNA [147] and ribosomal RNA synthesis,[148] the synthesis of 7-deazaadenosine brominated at both the 7 (**59**) and the 8 (**57**) positions has been reported and other halogenated analogues have been studied.

Gerster and co-workers in 1967 [149] published a route which involved the bromination of the fully protected 6-chloro-7-deazaadenosine (**60**) with N-bromoacetamide in chloroform. Subsequent deprotection, and conversion of the 6-chloro group to 6-amino using methanolic ammonia, yielded 7-bromo-7-deazaadenosine (**59**). This method was rather involved and could not be adapted to yield the 8-bromo product.

Subsequently a much more simple method has been reported by Bergstrom and Brattesani.[150] They used the mild brominating reagent and solvent combination of N-bromosuccinimide with DMF, [151] to directly brominate unprotected 7-deazaadenosine (**58**). By a careful study of this reaction Bergstrom and Brattesani were able to brominate selectively at the 7-position (**59**) with a small amount of 7,8-dibromo-7-deazaadenosine (**61**) formed as the by-product.

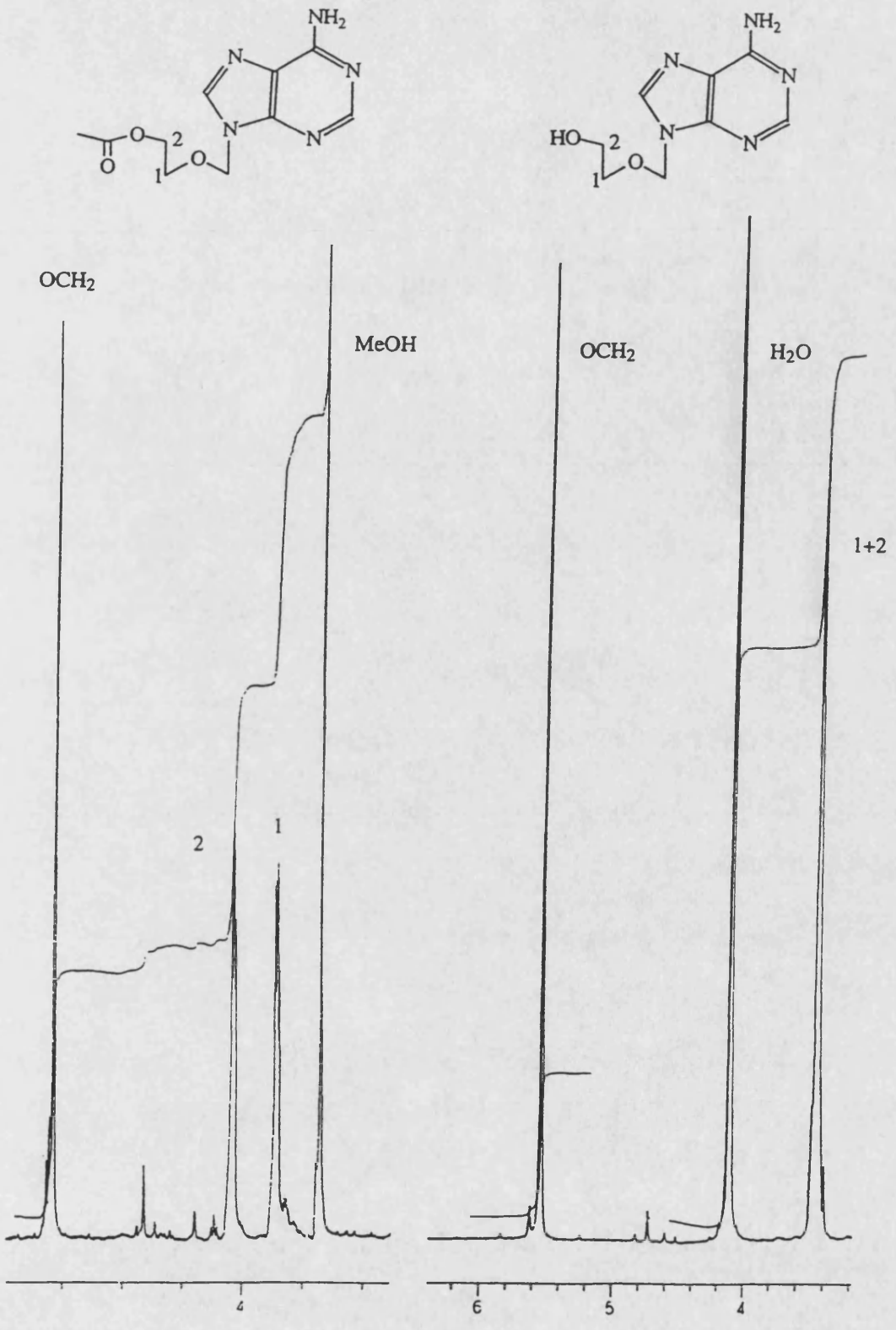


Figure 2.8 Part of the ^{1}H NMR Spectra of 9-[(2-Acetoxyethoxy)methyl] Adenine (**56**) and 9-[(2-Hydroxyethoxy)methyl] Adenine (**49**) Showing the Positions of the CH2 Groups (270MHz, d6DMSO).

Importantly though, when the reaction mixture was buffered with potassium acetate the major product could be altered to be 7-deaza-8-bromo-adenosine (**57**) with only a small amount of the 7-bromo-7-deazadenosine (**59**) and 7, 8-dibromo-7-deazadenosine (**61**) being produced. This method was repeated successfully to synthesise 7-deaza-8-bromo-adenosine in 40% yield.

The alteration in position of bromination depending upon the reaction medium can be explained as follows. Electrophilic substitution of the heterocyclic ring using N-bromosuccinimide results in the formation of a σ complex with a carbonium ion adjacent to the site of bromine addition (figure 2.9).

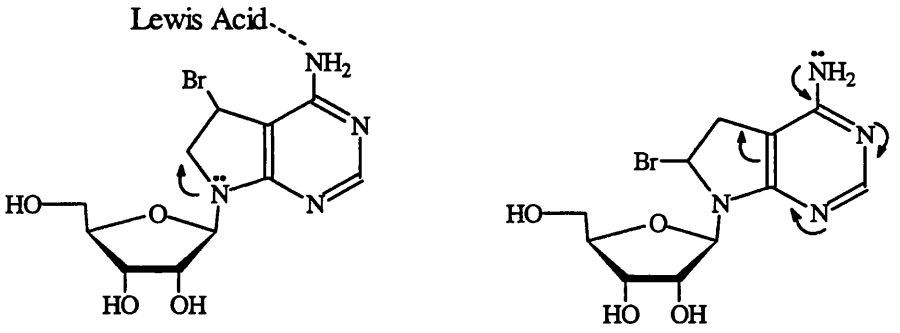


Figure 2.9 Stabilised Carbonium Ion Intermediates in the Bromination of 7-Deazaadenosine (**58**).

In general, the amino group is complexed by Lewis acids, generated from N-bromo succinimide, and is therefore unable to donate electron density via the π system to stabilise this carbocation. Thus the positive charge can only be stabilised on the 8 position by conjugation with the lone pair of the N9 atom resulting in substitution at C7. However, when potassium acetate buffer is present, the amino group is prevented from

complexing with Lewis acids and is therefore now able to stabilise the carbocation at C5 via the π system with resultant electrophilic substitution at C8.

When purine rings are substituted with bromine at the 8-position, the free rotation which normally exists about the glycosidic bond is hindered by the steric bulk of the bromine atom. The ring is now fixed in what is described as the high anti conformational form. As a result of this the H2′ proton resonance in the ^{1}H NMR is shifted downfield to 5.1ppm compared with an expected frequency of 4.5ppm in the non-substituted compound (figure 2.10).

2.6 Synthesis of 9-β-D-Purine 2′-Deoxyribofuranoside (62) and 9-β-D-Purine Ribofuranoside (63)

Although many synthetic procedures exist for the synthesis of purine ribofuranosides the most direct route is the reductive deamination of adenosine analogues.[152] Conventional deamination reagents (eg diazotization with nitrous acid followed by dediazoniation with H3PO2 [153]) cannot be utilised in the case of adenosine since the intermediate diazonium compound is attacked by water to yield inosine (**64**).[154] However, it has been reported that aromatic amines can be deaminated in one step by treatment with an alkyl nitrite in boiling THF, [155] and it was a modification of this method which was used to deaminate adenosine.

Initially, it was necessary to protect the ribosyl hydroxyls by peracetylation, to prevent the formation of nitrite esters of the sugar in the subsequent step. For both adenosine (**20**) and 2′-deoxyadenosine (**65**) this was achieved using acetic anhydride in pyridine.[156] Recrystallisation of the products from MeOH yielded 2′,3′,5′-tri-*O*-acetyl adenosine (**66**) and 3′,5′-di-*O*-acetyl 2′-deoxyadenosine (**67**) in 90% and 65% yield respectively. The acetylated product also had an enhanced solubility in THF solvent.

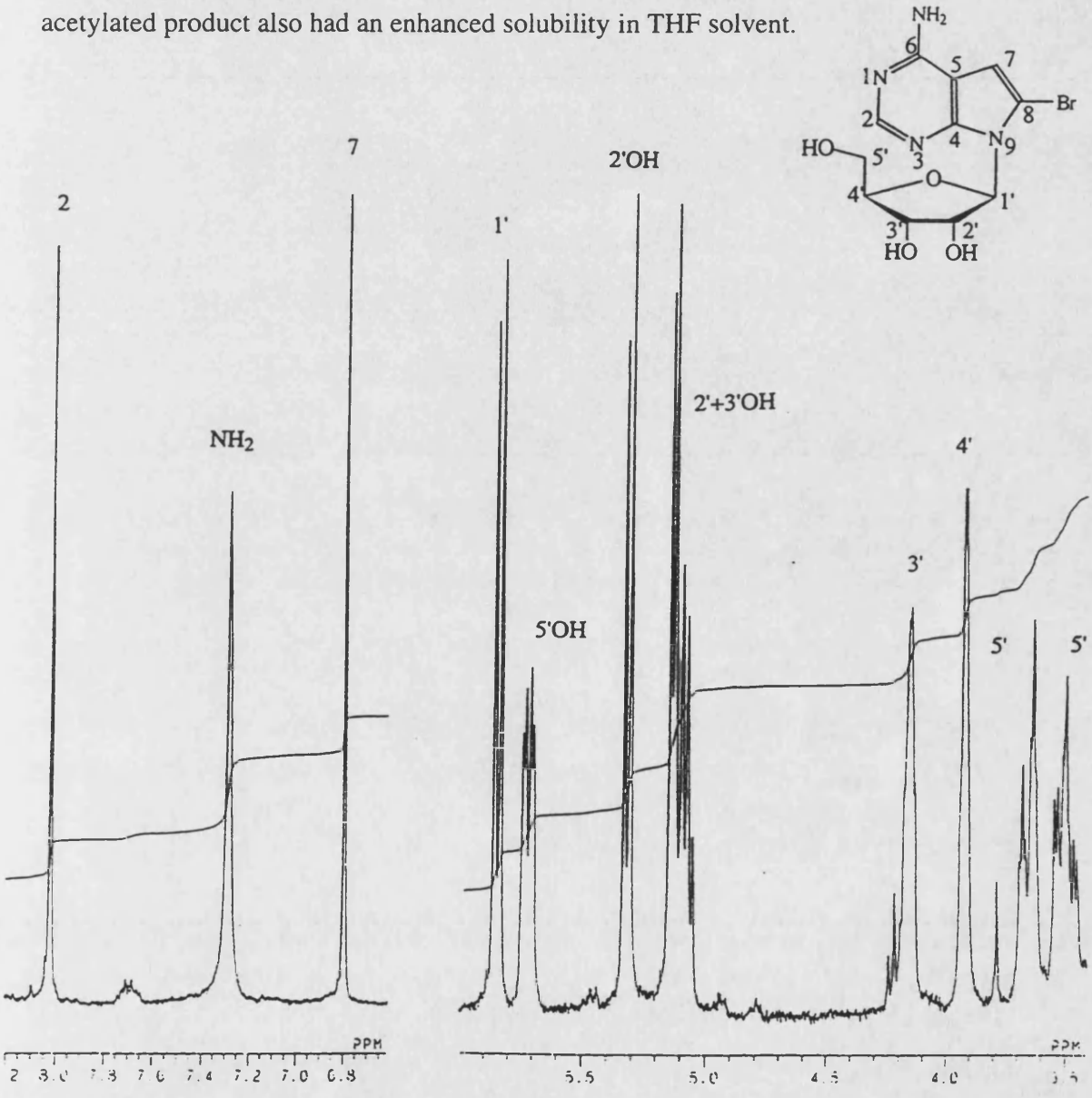
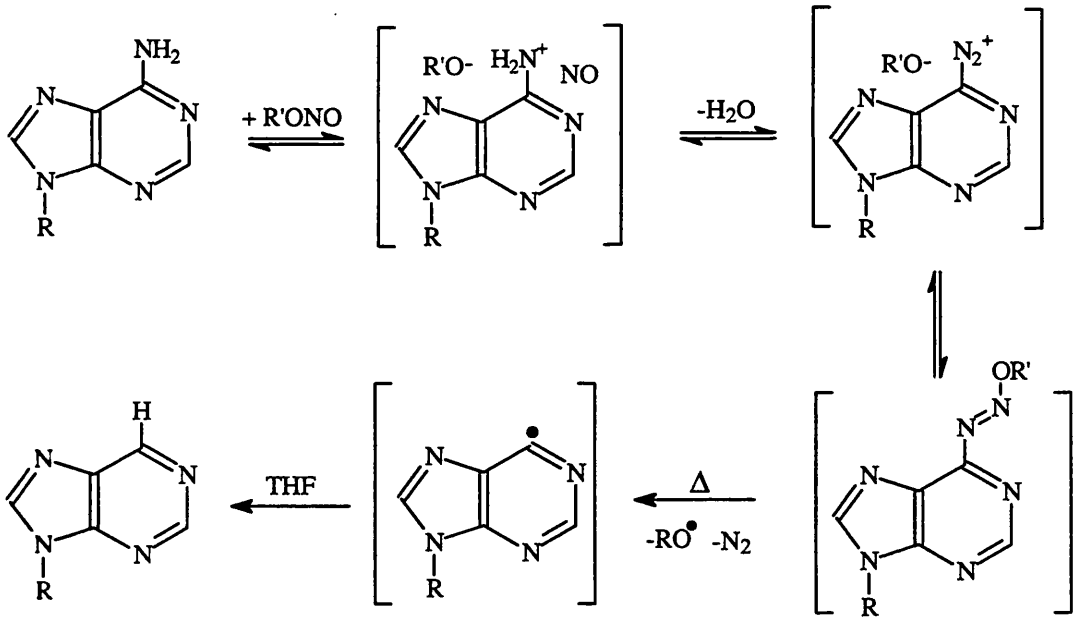


Figure 2.10 Part of the ^{1}H NMR Spectrum of 7-Deaza-8-Bromo-Adenosine (**57**) Showing the Down-field Shift of the 2′H (270MHz, d6DMSO).

n-Pentyl nitrite, the reagent for deamination, was carefully prepared from acidifed pentanol and sodium nitrite.[157] It was purified by distillation, stored in the dark and used within two weeks. The acetylated products from the previous step were reacted with the *n*-pentyl nitrite in dry THF for 4 days at 50°C.[152] The 6-amino group reacted with the *n*-pentyl nitrite to form the 6-diazonium salt which existed in equilibrium with the corresponding azo compound (figure 2.11). Both heat and certain frequencies of light homolytically dissociate such salts to yield aryl radicals,[152] which subsequently abstract a hydrogen atom from THF to yield the purine nucleoside. The two other radicals produced in the reaction, namely from the *n*-pentyl nitrite and from the THF, are almost certainly quenched by a chain termination reaction with another like radical.



R - 2', 3', 5'-tri-O-acetyl ribose or 3', 5'-di-O-acetyl 2'-deoxyribose
R' - *n*-pentyl nitrite

Figure 2.11. Mechanism for Deamination of Adenosine.

After work up, the products were purified by flash chromatography to yield 9-β-D-purine 2′, 3′, 5′-tri-*O*-acetyl-ribofuranoside (**68**) and 9-β-D-purine 3′, 5′-di-*O*-acetyl-2′-deoxyribofuranoside (**69**) in 65% and 55% respectively. A small amount of protected inosine by-product (**70**) was obtained when synthesising 9-β-D-purine 2′, 3′, 5′-tri-*O*-acetyl-ribofuranoside and was thought to have resulted from residual water in the *n*-pentyl nitrite.

Deprotection of the hydroxyl groups was readily accomplished by further reaction with ethanolic ammonia, a standard deprotection procedure.[123] Reaction for 2-3h yielded 2′-deoxy compound (**62**) with excellent yield (90% crude) but deprotection of 9-β-D-purine 2′, 3′, 5′-tri-*O*-acetyl-ribofuranoside was less successful, 18% yield of the deprotected product (**63**). This low yield was consistent with that reported by Nair and Richardson (71% and 32% repectively) for the same reactions.[152] Both the products were purified further by recrystallisation from EtOH and fully characterised.

The NMR spectra of these compounds illustrate clearly some features common to NMR spectra of nucleosides:

(i) the deamination is clearly shown with the appearance of a new aromatic resonance at 9.1ppm (figure 2.12). The unambiguous assignment of these aromatic peaks is known, having been assigned using specifically deuterated derivatives,[158] and the relative positions of the peaks are H6, H2, H8 in decreasing order of chemical shift.

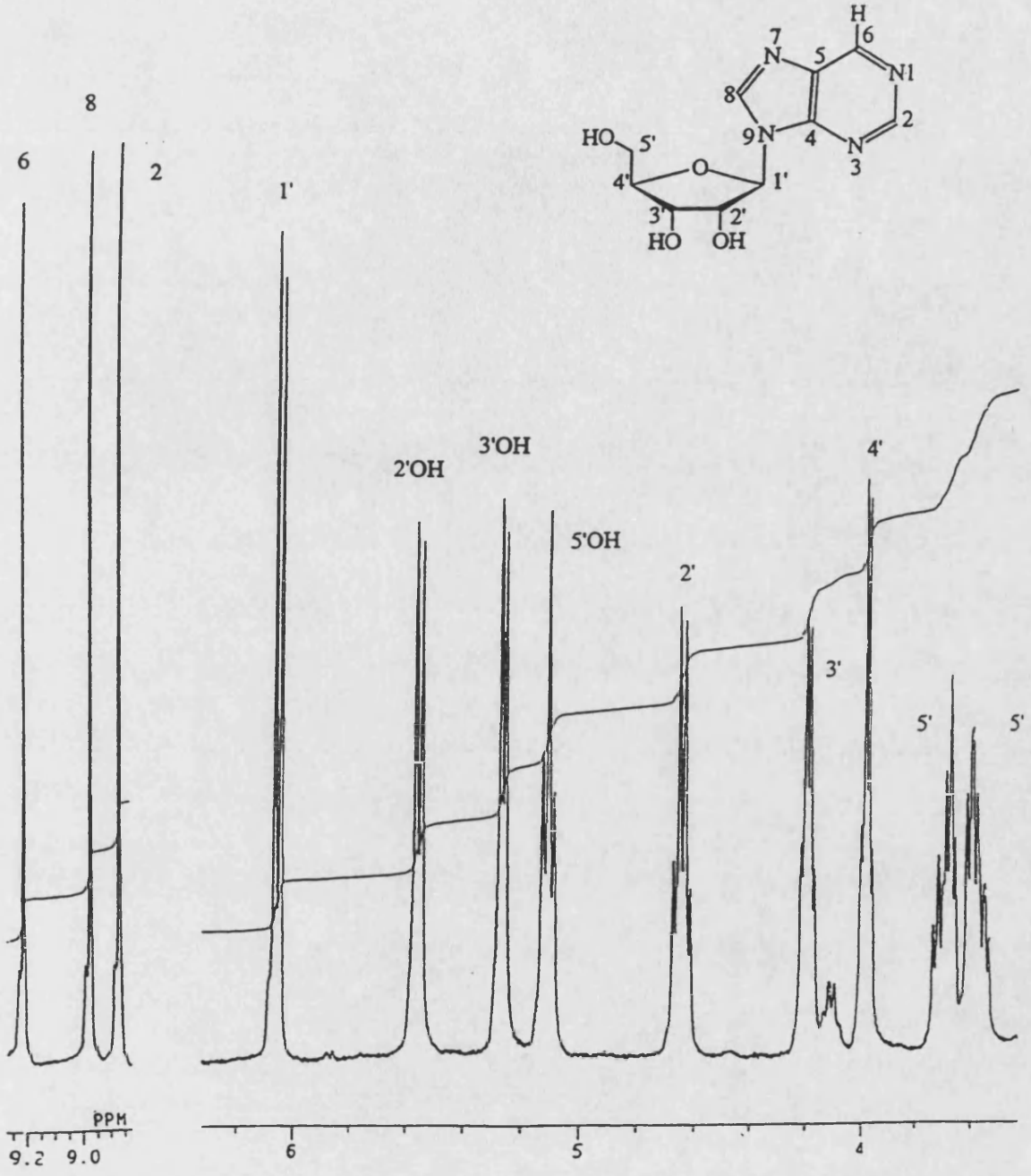


Figure 2.10 Part of the ^{1}H NMR Spectrum of 9-β-D-Purine Ribofuranoside (**63**) Spectrum (400Mz, d6DMSO).

(ii) the H1′ proton, or the anomeric proton, appears as a doublet at approximately 6ppm. From knowing the position of this proton a 2D COSY NMR can be used to readily assign all the other ring protons (figure 2.13).

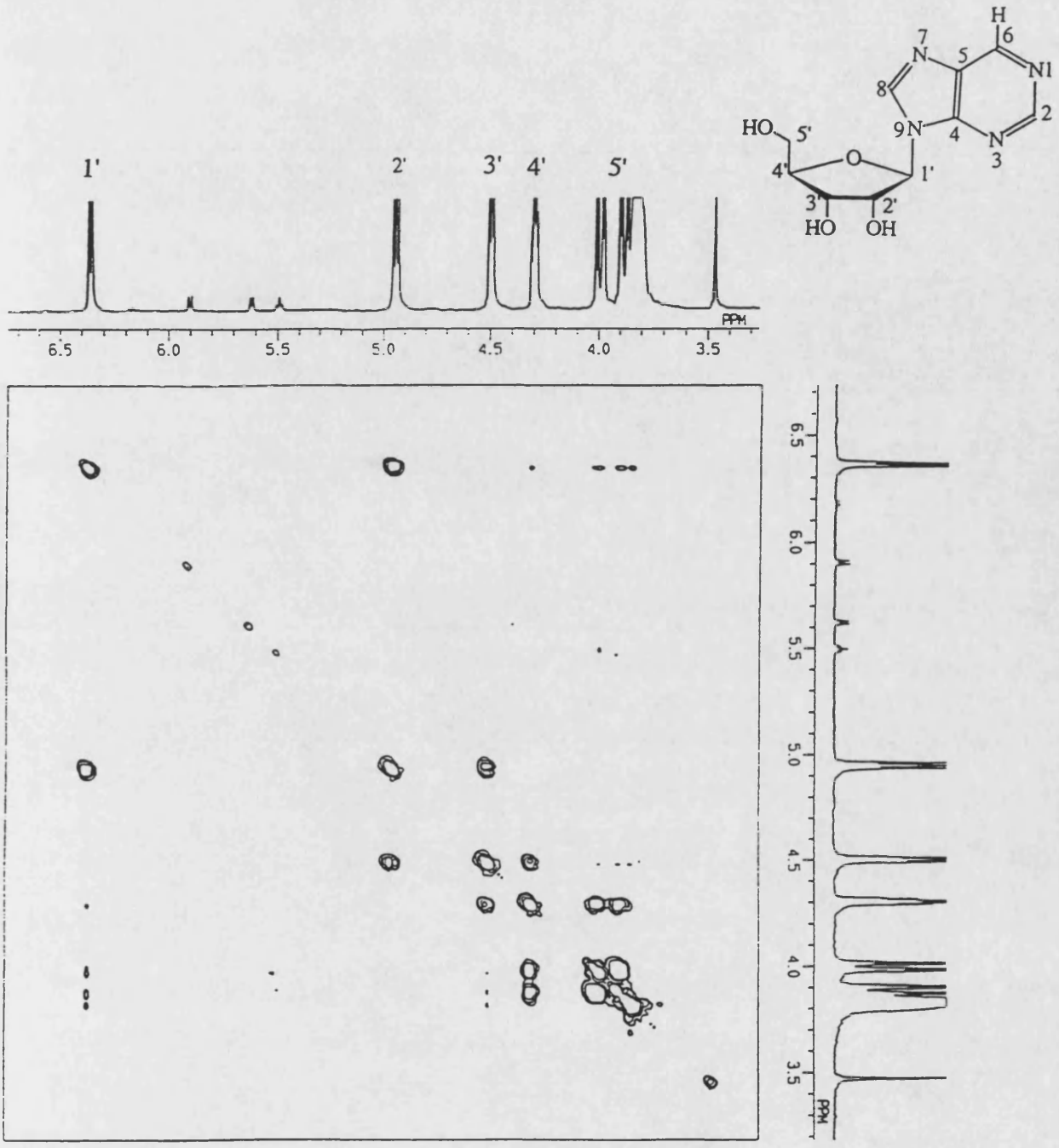


Figure 2.13 Part of a ^{1}H ^{1}H COSY NMR Spectrum showing the Ribose Protons - the Hydroxyls had been Exchanged with D2O Shake (400MHz, d6DMSO; this material is contaminated with a small amount of ribose and was recrystallised again before use in further synthesis).

(iii) in the 2′-deoxy compound the protons at the H2′ position could be accurately assigned as being α or β by calculation of the coupling constants. The Karplus equation defines the relationship that exists between the dihedral angle between two protons and the size of their coupling constant.[159] When the dihedral angle is 180°, i.e. when the protons are diaxial (or trans-trans) to each other, then the coupling constant is larger than when the protons are either equatorial-trans or equatorial equatorial to each other. H3′ and H2′α have a diaxial stereochemical relationship and thus will have the larger coupling constant.

2.7 Synthesis of 9-β-D-Purine 2′, 3′-*O*-Isopropylidene Ribofuranoside (71)

The 2′ and 3′ OH groups of 9-β-D-purine ribofuranoside were protected using an iospropylidene acetal using the experimental procedure described for adenosine (section 2.2). This gave the product in 79% yield as an oil which could not be induced to crystallise.[160]

CHAPTER THREE

SYNTHESIS OF NUCLEOTIDES

3.1 Introduction

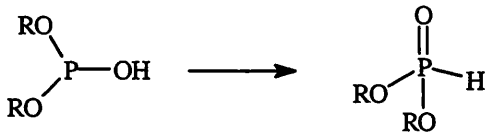
The following chapter describes the methods used to achieve selective phosphorylation of the nucleosides analogues discussed in the previous chapter. It includes a review of the development of phosphorylating agents and a discussion of the methods used and the phosphates synthesised.

The nucleotide 5'-monophosphates, eg AMP (**19**), have many fundamental biological roles (section 1.9.1) and thus the synthesis of such phosphate esters has been extensively studied. To date, many varied methods have been employed for the phosphorylation of both purine and pyrimidine nucleosides. Unfortunately though despite this, the literature is difficult to follow, as much of the work was performed in the 1950's and uses very old techniques. Papers describing more recent syntheses of nucleotides often fail to give full experimental detail, with the reactions seeming to depend largely upon the quality of the reagents and giving variable results. A brief overview of the major phosphorylation methodology is given *vide infra* (section 3.2).

Like carbon, phosphorus occupies a fairly central position in the periodic table and is able to form highly covalent bonds with both electronegative and electropositive elements resulting in a varied inorganic and organic chemistry. The ground state electronic configuration of phosphorus can be expressed as $1s^2 2s^2 2p^6 3s^2 3p^3$ and in the organic field, the chemistry of the P(III) and P(V) oxidation states predominate. Like nitrogen, it readily forms compounds with three sp-hybrid covalent bonds. These compounds, which are tetrahedral in shape with the fourth electron pair in a lone pair orbital, can act both as

nucleophiles, with the lone pair attacking an electrophilic centre, and also as electrophiles, with the incoming nucleophile attacking the phosphorus atom. As electrophiles they are very reactive, partly as a result of the low lying d-orbitals allowing the stabilisation of a greater number of bonds, and also because of the lack of steric crowding about the phosphorus atom.

The P(V) oxidation state results from the 3p and 3d orbitals lying close in energy enabling them to hybridise and allow phosphorus to form compounds with 5 covalent bonds. Such compounds can be divided into two groups, those which do and do not contain a P=O bond. All P(V) compounds react as electrophiles and if they do not contain a P=O bond often give products which do as a result of its high thermodynamic stability (section 1.9.1). An example of this bond strength being utilised chemically is the observation that phosphites rapidly tautomerise to give H-phosphonates (figure 3.1).



phosphite H-phosphonate

Figure 3.1 Tautomerisation of Phosphite Esters to H-Phosphonate.

A good overview of the organic chemistry of phosphorus has been written by Walker.[161]

The only naturally occurring isotope of phosphorus is ^{31}P which has a nuclear spin of I=1/2, and hence NMR spectroscopy can be used to study these compounds. In particular, the fine structure of ^{1}H ^{31}P coupled spectra is useful in determining the nature of the group

directly bonded to the phosphorus. In our work, for example, this was used to confirm that the phosphate was bonded to the 5' hydroxyl and not the 2' or 3' position (figure 3.2). There are however some practical difficulties with ^{31}P NMR spectroscopy resulting from the fact that the relative sensitivity for ^{31}P nuclei is very much lower than the sensitivity for ^{1}H. Hence, a high concentration of sample is also needed to acquire a good spectrum. Pulse delays also need to be longer than for ^{1}H to allow full recovery of the spins. Thus, when working in a field such as nucleotide chemistry, where the amounts of sample are often small, it can be difficult to obtain good ^{31}P spectra. In a similar manner to ^{1}H NMR spectroscopy, the chemical shifts of phosphorus resonances, which are measured relative to orthophosphoric acid, are indicative of the species present.

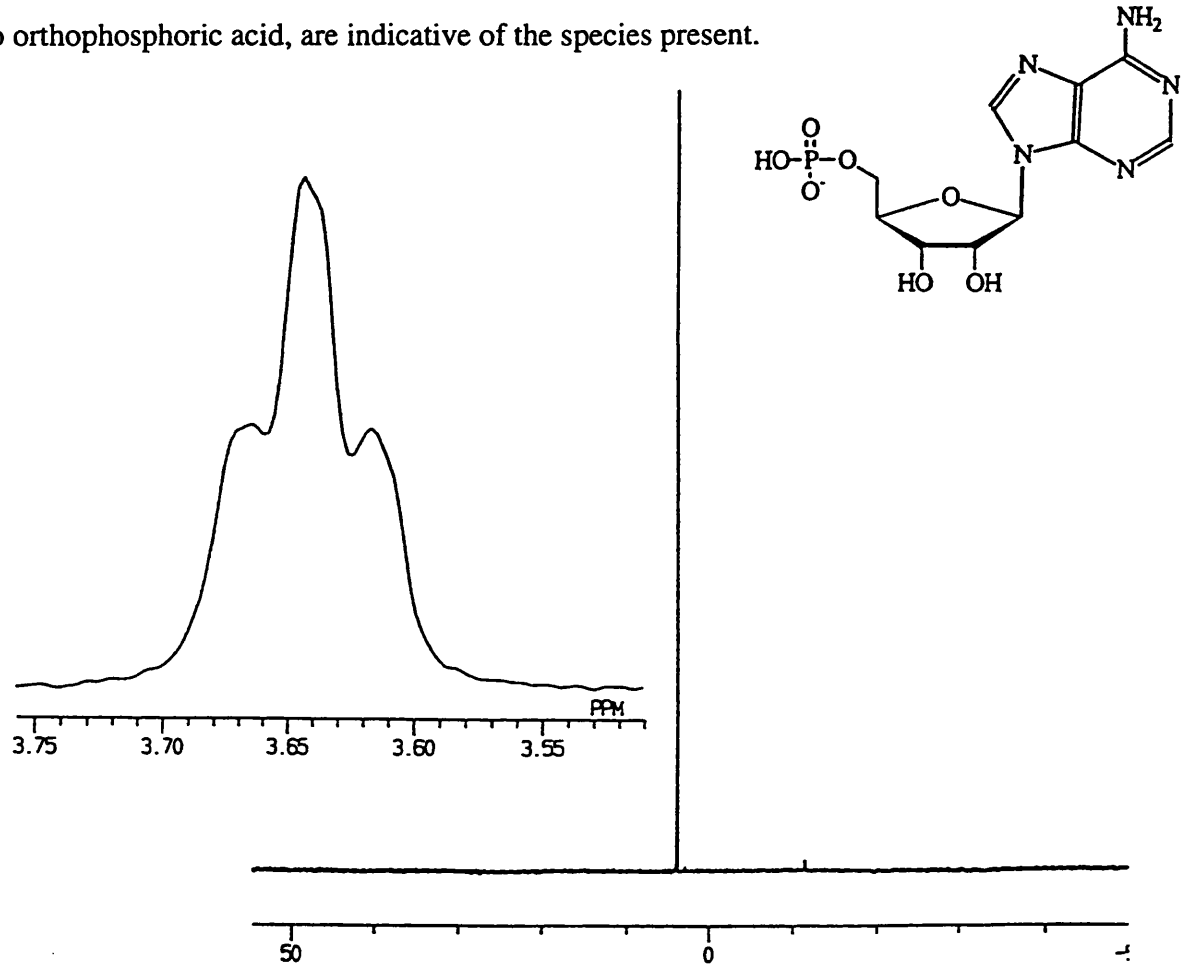


Figure 3.2 Typical ^{31}P NMR of a Nucleotide Showing the ^{1}H-^{31}P Coupled NMR (inset) (161MHz, solvent D_2O).

For phosphate esters the chemical shift ranges from between +5 and -3ppm and is dependent upon the pH of the sample. Thus, whilst chemical shifts for the products are given in the experimental detail they are not definitive and can vary quite considerably.

The predominant forms of phosphorus in biological systems are as esters, anhydrides and amides of $PO(OH)_3$, orthophosphoric acid. Nucleotides are an example of a monoester of $PO(OH)_3$ with two remaining ionisable OH groups. The pK_a values of these hydoxyls are approximately 1.6 and 6.6 and thus at physiological pH there is an equilibrium between the monoanion and the dianion. Like carbon based esters, phosphate esters are susceptible to acidic hydrolysis and the rate at which this occurs is dependent upon whether it is a mono-, di- or triester. An essential feature for the stability of DNA, RNA and 3', 5'-cAMP is that these esters are hydrolysed very slowly. The relatively rapid hydrolysis of the monoesters proceeds via the conjugate acid at ambient and low pH (figure 3.3).

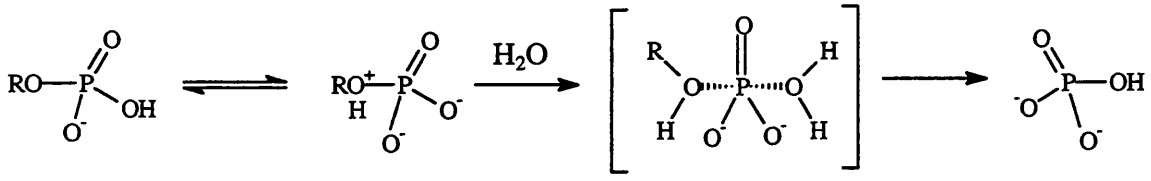


Figure 3.3 Neutral Hydrolysis of Phosphate Monoesters.

All these esters are resistant to alkaline hydrolysis.[162]

3.2 Methods of Phosphorylation of Purine Nucleosides

The synthesis of adenosine-like purine nucleotides can be viewed in two ways (figure 3.4) - either as the nucleophilic attack by an oxygen atom of a phosphate anion on carbon with the concomitant displacement of a suitable leaving group such as a halide or, more commonly, the phosphorylation of a hydroxyl group with an active phosphorus-containing compound.[163]

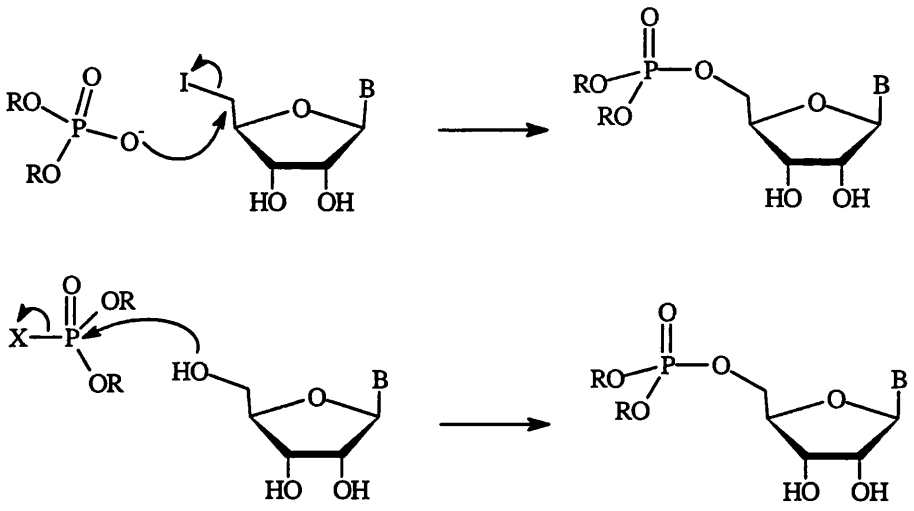


Figure 3.4 Methods of Synthesis of Phosphate Nucleotides.

Due to the difficulty of preparation of suitable substrates for the first method it is seldom encountered, although monohydrogen tetra-*n*-butyl ammonium salts of diphosphate have been used to successfully displace 5′-tosylates of adenosine.[164] The second method, however, is not without its problems since the nucleoside to be phosphorylated generally has three hydroxyl groups on the ribose ring, all of which are able to act as nucleophiles.

Although, in general, primary hydroxyl groups are more reactive than secondary ones, this reactivity differential alone is not large enough to be utilised for regioselective control.

In the cell nucleosides are phosphorylated by enzymes called kinases.[165] These enzymes have been isolated and utilised by chemists for phosphorylation. The advantage of this method is that problems of regio- and stereo-specificity are automatically controlled by the enzyme, but the implicit disadvantage of such a method, is that synthetic nucleosides may not be substrates for the enzyme.[166]

A second synthesis of nucleotides activates a monoalkyl phosphate with *N*, *N'*-dicyclohexyl carbodiimide (DCC) to give an intermediate imidoyl phosphate. This is in turn reacted with a nucleoside protected such that only one hydroxyl remains free [167] (the mechanism of DCC coupling is shown, section 4.2.3). Subsequent deprotection of the hydroxyls and the phosphate yields the nucleotide product.

The requirement for initial activation by DCC was obviated by the development of phosphorylating agents with an intrinsic leaving group. The phosphorochloridates are ideal for this purpose, but are unselective, so it is necessary to protect both other hydroxyls and the phosphate. The early syntheses using this method protected the phosphates with benzyl ethers, for example dibenzyl phosphochloridate [168] or *p*-nitrophenyl esters,[169] groups which could be removed by hydrogenolysis or strong alkali respectively. More recently, protection with β-cyanoethyl groups, which are readily removed by the use of dilute alkali (section 3.5), has led to one of the most widely used phosphorylating reagents.[170] This makes the reagent suitable for the preparation of

nucleotides which may be sensitive to more severe deprotection methods. In general the phosphorochloridate methodology is still appropriate when selectively protected phosphates are required.[171]

However, there was still a need for a phosphorylating reagent which could regioselectively phosphorylate the 5'-hydroxyl function. The most simple phosphorochloridate, $POCl_3$ itself, had not been widely used for phosphorylation since it gave low yields and no specificity of reaction. Initially, Yoshikawa and Kato [172-173] demonstrated that its action could be improved when used neat for the phosphorylation of 2', 3'-*O*-isopropylidene nucleosides. However, the observation that, by using a trialkyl phosphate solvent and only a slight excess of $POCl_3$, a variety of unprotected nucleosides can be phosphorylated selectively at the 5'OH has made this method extremely useful.[174] This method is discussed in more detail later (section 3.3).

Other methods for the 5'-phosphorylation of nucleosides utilise sterically hindered phosphorylating agents to achieve regioselectivity. The method of Hes and Mertes [175] uses di(2-tert-butylphenyl) phosphorochloridate to phosphorylate thymidine and guanosine selectively in 70% and 55% yield respectively (figure 3.5A). Similarly a selective phosphorylating reagent has been achieved by the reaction of phosphoryl chloride with a mixture of acetonitrile, water and pyridine before addition of the nucleoside.[176] The proposed reactive intermediate is shown below (figure 3.5B).

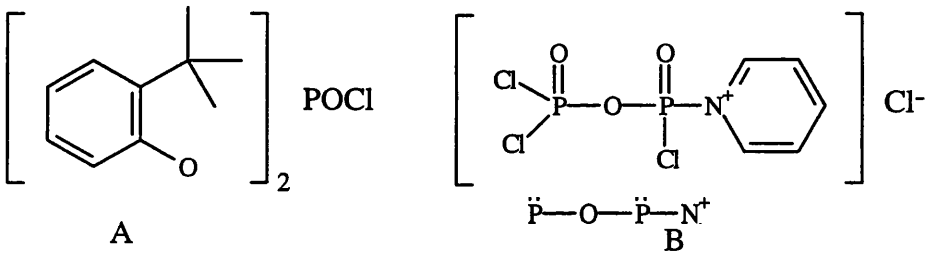


Figure 3.5 Sterically Hindered Regioselective Phosphorylating Agents.

It is of interest to note that, despite there being several procedures, phosphorylation is generally not considered commercially viable and major chemical companies still obtain their nucleotides, where possible, by degradation of DNA or RNA or by use of enzymatic methodology. It is for this reason that there are surprisingly few nucleotides available for purchase and those which do not occur naturally with great abundance are very expensive, eg 7-deazaadenosine 5'-monophosphate costs £151.80 for 50mg.[177]

3.3 Synthesis of the Nucleotides

With the overall project in mind, it was ideal that phosphorylation of nucleosides could be achieved with the minimum of protection. For this reason, in general, the $POCl_3$ method [174] was used to achieve selective phosphorylation. A mixture of dry nucleoside was heated in triethyl phosphate to 50°C for 15minutes, cooled to 0°C and $POCl_3$ added. When the reaction was complete it was quenched by the addition of iced water and the product purified to give the nucleotide (figure 3.6).

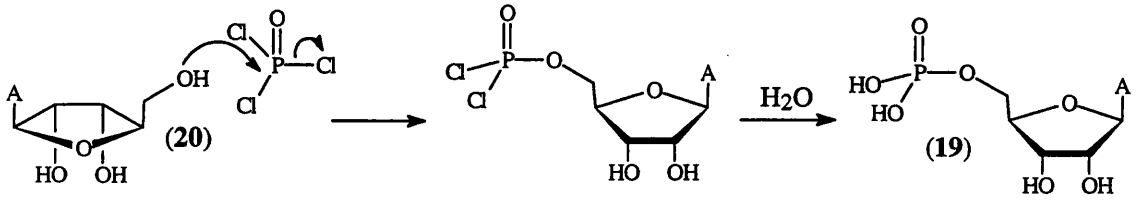


Figure 3.6 General Phosphorylation Mechanism.

The selectivity of this reaction arises from the initial mixing with trialkyl phosphate although until recently the exact reason as to why this occurred was unknown. Initially it was suggested that the trialkyl phosphate caused an *in situ* protection of the cis diol [178] and then it was proposed that the trialkyl phosphate was able to react with $POCl_3$ to form a sterically hindered reactive intermediate.[179]

$$(EtO)_3PO + POCl_3 \rightarrow [(RO)_3P\text{-}O\text{-}POCl_2]^+ Cl^-$$

However, in 1995 Ikemoto *et al.* reported a close investigation of this mechanism.[180] By heating guanosine with triethylphosphate, and then cooling the mixture, they were able to isolate a complex, which further reacted regioselectively with $POCl_3$. Using elemental analysis, mass spectra and infra-red analysis this species was shown to consist of a 1:1 ratio of the nucleoside and triethyl phosphate, but the P=O and C=N stretching frequencies were at lower wavenumbers when compared to the values in a straightforward mixture of the two components. This indicated an interaction between the P=O group and the N7 of the purine ring.

It is known that nucleosides exist in three possible conformations as regards the rotational position of the purine ring relative to the sugar, the *anti*, *high-anti* and *syn* forms. For

guanosine the presence of both the *anti* and *high-anti* forms has been confirmed by X-ray analysis,[187] (figure 3.7) and the two conformations show some differences in peak frequencies in solid state NMR.

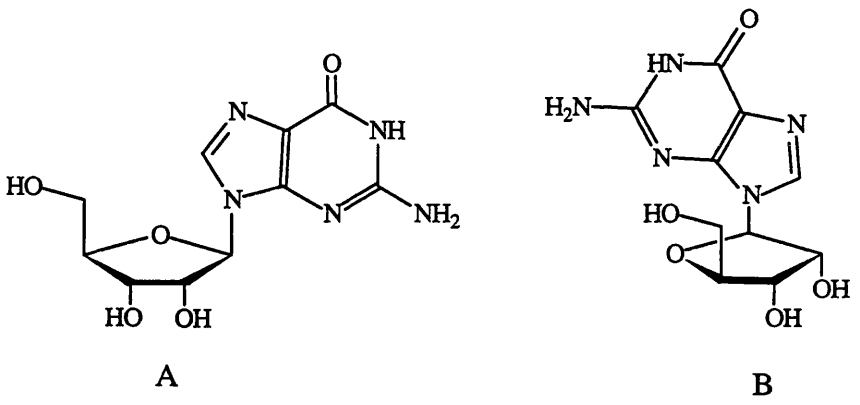


Figure 3.7 The (A) *Anti-* and the (B) *High-Anti-* Conformations of Guanosine.

Interestingly NMR analysis of the isolated triethyl phosphate - guanosine complex showed only those signals arising from the *high-anti* conformation, and a complex was proposed as this intermediate (figure 3.8).

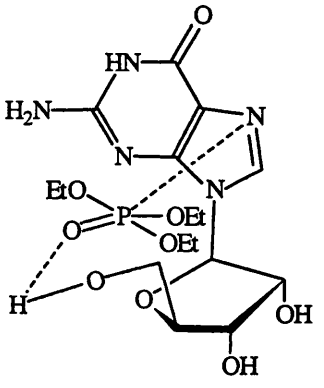


Figure 3.8 Complex Formed Between Triethyl Phosphate and Guanosine.

The formation of a hydrogen bond between the 5'OH and the triethyl phosphate is thought to activate this position to nucleophilic reaction and, as a result, the regioselective

phosphorylation with $POCl_3$ is possible. The rate of this phosphorylation therefore depends both upon the relative nucleophilicity of the nitrogen of the purine ring and the conformation of the nucleoside allowing the complex to form easily, and thus reaction times vary considerably between different nucleosides. Although this is the most recent theory proposed to explain the regioselectivity of this mechanism the situation is obviously not simple. Within this project nucleosides with no nitrogen atom at the 7-position were phosphorylated with yields similar to those obtained for nucleosides with a nitrogen at this position, namely 7-deazaadenosine (**58**) and 7-deaza-8-bromo-adenosine (**57**). It is possible that in these examples the 5′OH is activated by a similar complex between the triethyl phosphate and a N atom at another position in the base ring for example the 3 position or the 1 position. Similarly, for this complex formation to be the case, heating is required to form the intermediate, this was not always the case when phosphorylation reactions were performed in this work. It is therefore still unclear as to why the use of triethyl phosphate confers regioselectivity to this reaction mechanism.

Although, in some cases, phosphorylation was followed by HPLC this was not always possible due to the retention times and so reactions were often worked up before they had gone to completion. Addition of water to the reaction mixture quenched the phosphorylation and hydrolysed unreacted P-Cl bonds. The drop in pH caused by the liberation of hydrogen chloride is often counteracted by the addition of a mixture of pyridine water.[182] Initially, a ratio of 4:1 water:pyridine was used but it was found to hydrolyse the P-Cl bonds too slowly allowing the intermediates to react repeatedly with any remaining $POCl_3$, resulting in the formation of polyphosphates which were not isolated. A ratio of 10:1 water:pyridine was found to be more effective with hardly any

by-products formed, but for reactions where the nucleoside was not particularly acid sensitive addition of an approximately 20 fold excess of iced water was found to be effective.A summary of all the 5' selectively phosphorylated products which were synthesised by this route is shown (tables 3.1A and B).

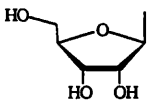
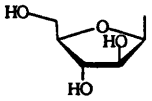
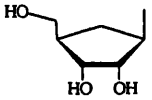
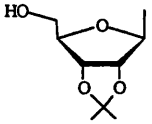
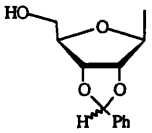
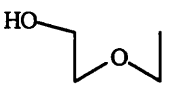
Sugar	Base	Product Number	Reaction Temperature, Time and Work Up	Isolation Method	Yield
	adenine	**19**	100°C, 3h iced water	ion exchange, acetone pptn.	39%
	adenine	**72**	50°C, 18h iced water	ion exchange, charcoal column, acetone pptn.	58%
	adenine	**73**	50°C, 18h iced water	ion exchange, charcoal column, acetone pptn.	56%
	adenine	**74**	50°C, 18h iced water	ion exchange, charcoal column, acetone pptn.	47%
	adenine	**75**	50°C, 1h iced water	amberlite XAD-4 column, ion exchange	14%
	adenine	**77**	50°C, 1.5h iced water	ion exchange, charcoal column, acetone pptn.	55%

Table 3.1A Phosphorylation of Analogues Modified in the Ribose Ring.

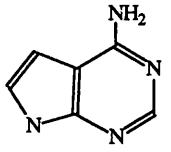
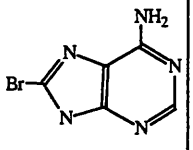
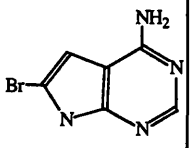
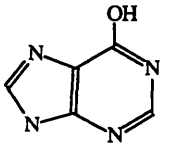
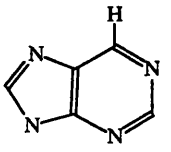
Sugar	Base	Compound Number	Reaction Temperature, Time and Work Up	Isolation Method	Yield
ribose		**77**	50°C, 18h iced water	ion exchange, charcoal column acetone pptn.	48%
ribose		**78**	100°C, 2.5h 3:1 water: pyridine	ion exchange, material was left impure	53%
ribose		**79**	50°C, 1.5h 9:1 water: pyridine	ion exchange	44%
ribose		**80**	100°C, 4h iced water	ion exchange, material was left impure	32%
2'-deoxy ribose		**81**	50°C, 24h 5:1 water: pyridine	ion exchange, charcoal column	no 5' reacted material

Table 3.1B Phosphorylation of Analogues Modified in the Purine Ring.

In general, this method [174] provided selective phosphorylation at the 5' position with little or no side products found - there were, however, two notable exceptions. The first was the phosphorylation of adenine 9-β-D-arbinofuranoside (**72**) where two products were isolated and the ^{1}H ^{31}P coupled spectrum indicated one to be phosphorylated at the 5' (58%) and the other at the 2' or 3' (4.5%) (figure 3.9). In this compound the 2' hydroxyl group has a β configuration and is thus on the same face of the ribose ring as the purine.

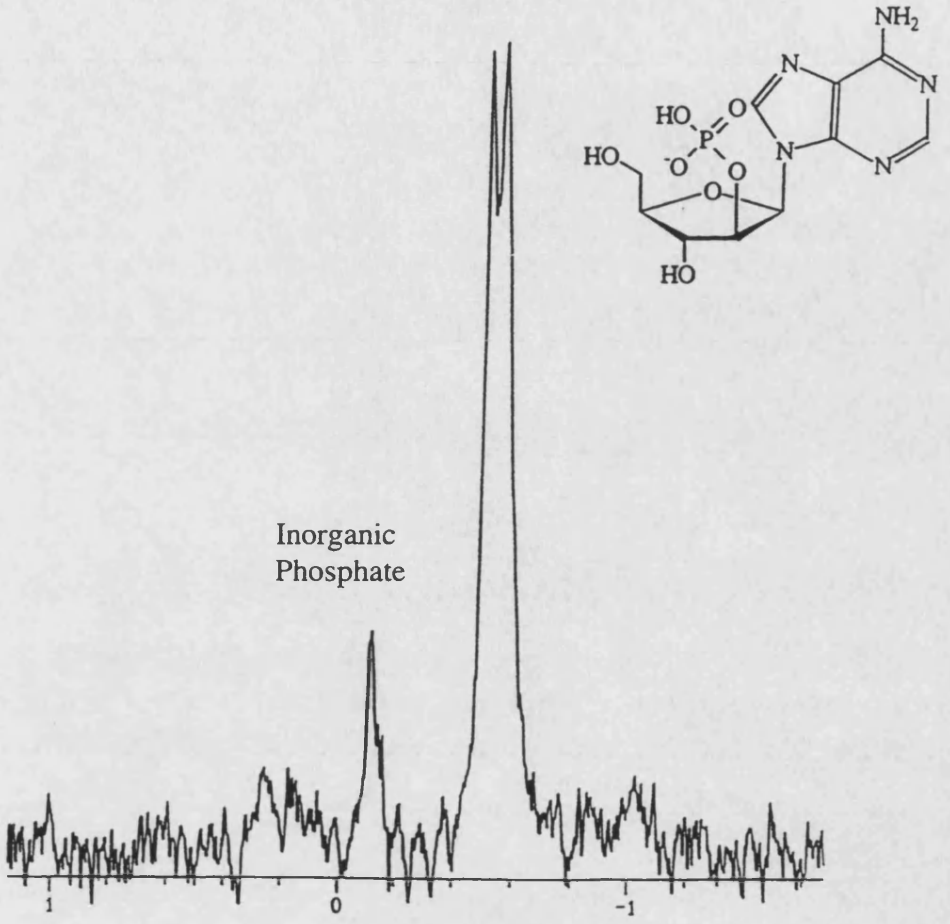


Figure 3.9 ^{1}H ^{31}P Coupled NMR Spectrum of Adenine 9-β-D-Arabinofuranoside 2′-Monophosphate (**138**) (36.3MHz, solvent D_2O).

It can therefore be assumed that triethyl phosphate was also able to activate the 2'OH, by forming a complex analogous to that formed with the 5'OH, and thus promoted the formation of the 2'-phosphorylated side product. It is also of interest to note that the ^{1}H NMR spectrum of the 5'phosphorylated product showed two peaks for each of the aromatic protons. It was assumed that these different shifts resulted from the restricted rotation of the adenine ring about the ribose adenine linkage, a result of the steric hindrance from the 2' β hydroxyl (figure 3.10).

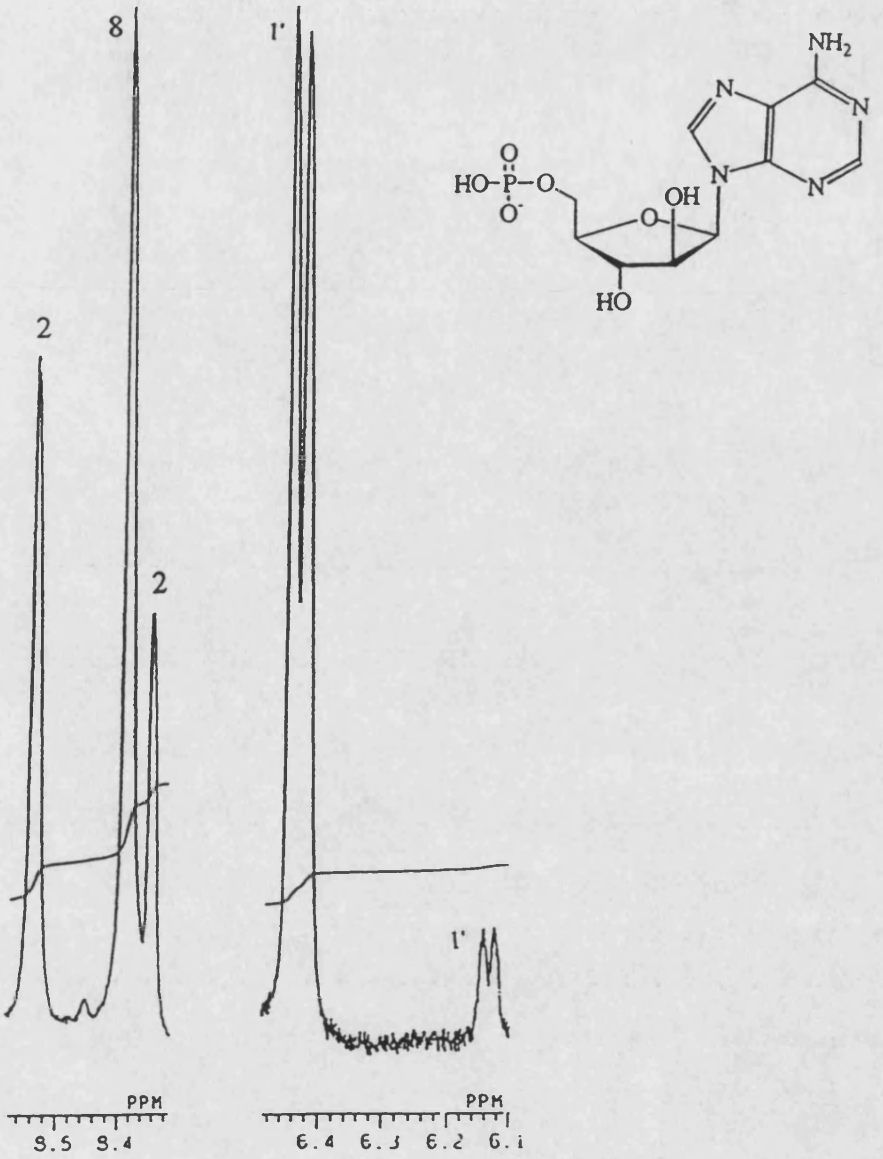


Figure 3.10 ^{1}H NMR of Adenine 9-β-D-Arabinofuranoside 5′-Monophosphate (**72**)

Showing the Aromatic and Anomeric Protons (270MHz, solvent D_2O).

Phosphorylation of the 9-β-D-purine 2'-deoxy ribofuranoside (**62**) synthetic nucleoside also provided particular problems and, despite several attempts, none of the 5' phosphorylated product (**81**) could be isolated with significant amounts of either the 3' phosphorylated material or polyphosphorylated products being formed. Interestingly, difficulties using this method to selectively phosphorylate unprotected 2'-deoxy nucleotides have been reported in the literature, with yields of the 3'-phosphorylated material by-product varying from 2-15%.[183] It was for this reason that P(III) chemistry was finally used to synthesise this particular purine nucleotide (section 3.5).

Phosphorylation of 2-hydroxyethoxy methyl adenine (**49**) did not require selective activation of any hydroxyl. It had been reported that 2-hydroxyethoxy methyl guanine (**83**) could be phosphorylated in 90-95% yield using *m*-cresol as the reaction solvent.[184] This method was attempted but, after reaction, HPLC analysis of the mixture showed a disappointing amount of product and, even after addition of further $POCl_3$, the yield could not be improved. The product was finally achieved in 55% yield by reverting to the triethyl phosphate method (figure 3.11).

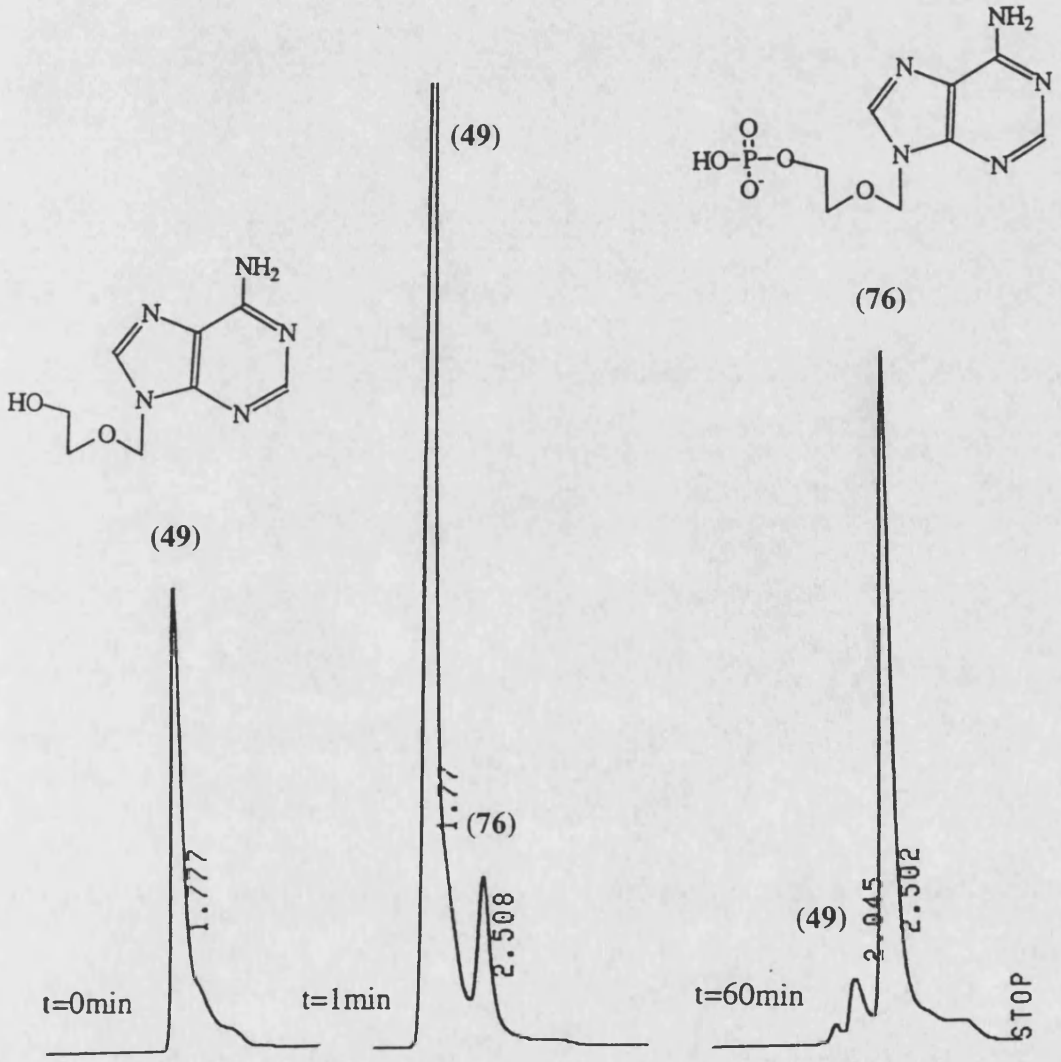


Figure 3.11 Following the Phosphorylation of 2-Hydroxyethoxy Methyl Adenine (**49**) Using HPLC (SAX column, 0.05M KH_2PO_4 /5%MeOH).

One way in which ^{13}C NMR spectra of nucleotides differ from that of the nucleosides is that ^{31}P ^{13}C spin spin coupling can be observed. For the two bond POC5' distance this coupling is roughly 5Hz [185] and was not always observed in the ^{13}C spectra of the nucleotides synthesised in this project. However, the size of a three bond POC5'C4' coupling can vary between 0-10Hz and is dependent upon the torsion angle of the chain in a Karplus-like relationship.[186] The ^{13}C NMR spectra of all the nucleotides synthesised in this project exhibited a P-C4' coupling constant of roughly 10Hz, indicating a antiperiplanar relationship of these two atoms with respect to each other (figure 3.12). This allowed the 4' carbon to be readily distinguished from the other furanoside ring carbon atoms in the ^{13}C NMR spectrum.

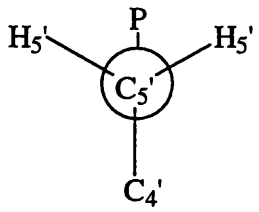


Figure 3.12 Antiperiplanar Relationship of the C4' atom with P in Adenosine - Like Nucleotides.

3.4 Purification of the Phosphate Product

The major impurity from direct unprotected phosphorylation methods is inorganic phosphate which results from the reaction not proceeding to completion. It is essential to remove this impurity since it interferes with the subsequent coupling reaction. Unfortunately, due to the pKa values of inorganic phosphate being similar to those of the nucleotide, the two phosphates are usually not separated by ion exchange chromatography using Sepharose Fast Flow resin and TEAB buffer. Therefore a subsequent purification

step was usually required. One notable exception was 7-deaza-8-bromo-adenosine 5'-monophosphate (**79**), in which the combined hydrophobic effect of the bromo group and the replacement of the N7 group with a CH, resulted in the phosphorylated product eluting from the ion exchange column at a higher concentration of buffer than in general (250-300mM as opposed to 150-220mM). As a result it was isolated pure from inorganic phosphate.

Methods previously reported for the removal of inorganic phosphate include its precipitation as the barium salt [187] or the precipitation of the nucleotide using acetone.[188] The use of barium salts was complex with a series of several precipitations required but, the precipitation of the nucleotide with acetone seemed to work adequately. For the best results it was necessary to have the proton or ammonium salt of the nucleotide, thus minimising its solubility in any residual water.

Another traditional method for cleaning up such monophosphates is the use of activated charcoal.[189] The nucleotide was adsorbed on to the charcoal, the inorganic phosphate removed by washing with MilliQ water and the desired product desorbed using dilute ethanolic ammonia. Recovery of the product was monitored by UV but was found to be inconsistent varying from 50% to 80%. UV analysis of the fractions also indicated that further material could not be recovered by excessive washing, approximately 70% of the material that would eventually be recovered was eluted in the first 300ml of solvent. Additionally, once strongly adsorbed, the nucleotide could not be desorbed either by using solvents with increased hydrophobic nature or those with increased ionic strength. For example, using *n*-butanol in place of ethanol provided no improvement in the recovery of

the product, neither did an increase in the % of ethanol or ammonia in the solvent. Thus it was important to minimise the irreversible adsorption of the nucleotides by keeping the size of the column to a minimum and by using a wide diameter column which allowed the solvent to flow through with a higher pressure. Hampton *et al.* [187] report adjusting the pH of the sample to pH=3.5 before loading onto the charcoal column, it is possible that this minimises irreversible adsorption.

After passing the nucleotide through a charcoal column subsequent alteration of the counter ion to tri-*n*-octylammonium was prevented by a contaminant which caused precipitation of the tri-*n*-octylamine. It was thought that the contaminant was an anion, possibly Cl^- or SO_4^{2-}, which had eluted from the charcoal column. The presence of chloride was tested for using silver nitrate [190] but this showed a negative result so the ion was thought to be sulphate. Ion exchange chromatography did not purify the nucleotide from this contaminant but acetone precipitation as described above was successful.

In summary, to purify the nucleotide product effectively it was in general necessary to first perform an ion exchange column to remove any unreacted material and other phosphate by-products, subsequently to use an activated charcoal column to remove the inorganic phosphate and then to precipitate the product with acetone and collect the pure material using centrifugation.

A more recent synthesis of nucleotides reported the use of a polystyrene Amberlite XAD-4 resin column to purify 3'-deoxy AMP (**84**) both from inorganic phosphate, which washed straight through the column, and unreacted nucleoside which had a longer

retention time than the phosphorylated material when the column was eluted with MeOH.[191] In theory, the adenine ring bound to the polystyrene resin by a π-stacking interaction between the heterocyclic ring and the phenyl resin rings. In our hands, phosphorylated adenosine did not bind to the resin and it was considered that the low aromatic nature of the adenine ring prevented formation of the π-π interaction. However, it was of interest to investigate if this method could be used for analogues of adenosine which contained a second aromatic ring. 2', 3'-*O*-Benzylidene adenosine (**45**) was phosphorylated and, when loaded onto the Amberlite resin, this nucleotide (**75**) bound. After washing the column with water, a gradient elution with aqueous MeOH eluted the product (**75**) freed from inorganic phosphate (figure 3.13).

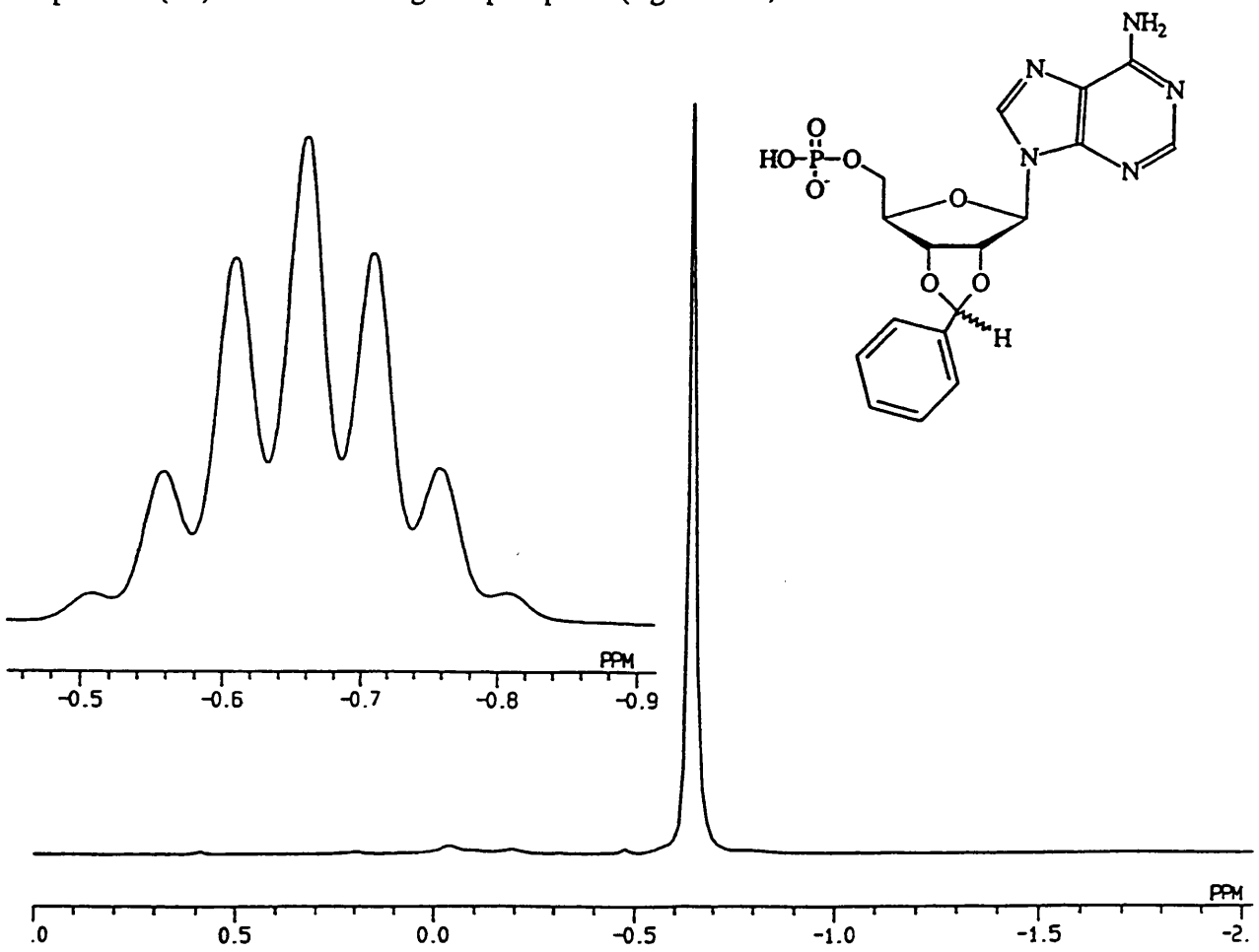


Figure 3.13 ^{31}P NMR of 2', 3'-*O*-Benzylidene Adenosine 5'-Monophosphate Showing the ^{1}H-^{31}P Coupled NMR (inset) (161.7MHz, solvent D_2O).

Disappointingly, the product was recovered in a poor yield but it was felt that this could be improved by use of a smaller column and a straightforward MeOH elution. However, in this case, the Amberlite XAD-4 resin did not separate the unreacted nucleoside from the nucleotide and a further ion exchange column was necessary. It is of interest to note that N1 phenyl AMP (**85**) is also able to bind to Amberlite XAD-4 resin in a similar manner (unpublished results from this laboratory). However what is clear is that, in our hands, adenosine itself is not able to bind to the resin. The interesting septet achieved in the ^1H coupled spectrum of this monophosphate was thought to be 2 AB quartets overlapping resulting from the two different isomers coupling with their adjacent 5′CH_2 groups.

3.5 Phosphitylation of Nucleosides

As discussed P(III) compounds, for example phosphite triesters, are very much more reactive then their P(V) analogues due to the presence of a lone pair (section 3.1), and such reagents have been utilised to synthesise phosphates by reaction with an alcohol followed by oxidation to give the P(V) adduct.[192] Although this reaction proceeds in general with high yields, the disadvantage of this method is that it is necessary for all the other nucleophilic centres of the alcohol reactant to be protected, and for the phosphite to also be protected. Despite this complication the phosphite triester approach was first developed for use with nucleosides for the synthesis of oligonucleotides in solid phase synthetic procedures [193] and this work has been so successful that the synthesis of such polymers has now been fully automated with the use of solid phase supports.[194]

This methodology was used to phosphorylate 9-β-D-purine ribofuranoside (**63**) for two reasons. Firstly phosphorylation using P(V) chemistry of 9-β-D-purine 2'-deoxy-ribofuranoside (**62**) had given many by products and secondly nucleoside protection was simplified as there was no N6-amino group. The 2' and 3' hydroxyls were easily protected using an isopropylidene acetal to give 9-β-D-purine 2′, 3′-*O*-isopropylidene ribofuranoside (**71**).

Phosphitylation of this fully protected nucleoside (**71**) was thus performed using tetrazole activated (biscyanoethoxy) (diisopropylamino) phosphine in dry dichloromethane (figure 3.14).

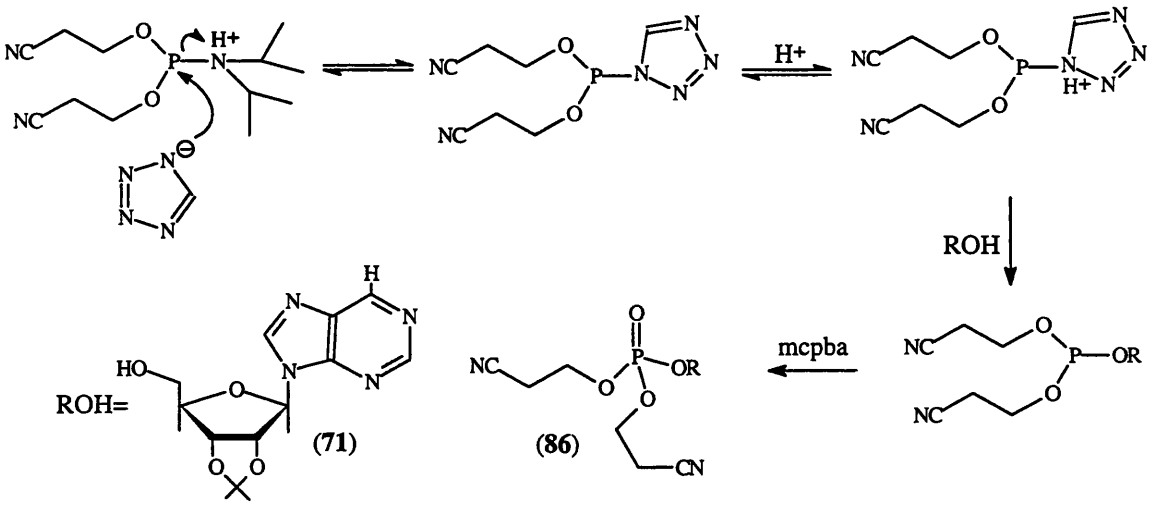


Figure 3.14 Reaction Scheme for the Phosphitylation of Alcohols.

Such reactions are readily followed by ^{31}P NMR. The phosphitylating agent had an initial resonance at 149ppm, which upon activation with tetrazole, moved to 128ppm. The fully protected nucleotide phosphate (**86**) had a resonance at -1.5ppm. Any water present in the reaction medium reacted rapidly with the activated reagent and then rearranged to give

phosphonate with a characteristic shift of 8ppm. This impurity, and a small amount of other material, was removed by flash chromatography to give the fully protected product (**86**) in 87% yield.

To prevent the formation of cyclic phosphates the cyanoethyl groups were removed first using concentrated ammonium hydroxide solution at room temperature.[195] These are cleaved by a β-elimination mechanism (figure 3.15) and gave 9-β-D-purine 2′, 3′-*O*-isopropylidene ribofuranoside 5′-monophosphate (**87**) in 29% yield.

Figure 3.15 Base Catalysed β-Elimination of 2-Cyanoethyl Groups.

The isopropylidene group was removed subsequently by acid catalysed hydrolysis [196] and the deprotection was monitored by HPLC. When complete 9-β-D-purine ribofuranoside 5′-monophosphate (**88**) was purified by ion exchange chromatography and isolated in 72% yield.

CHAPTER FOUR

SYNTHESIS OF NICOTINAMIDE DINUCLEOTIDE ANALOGUES

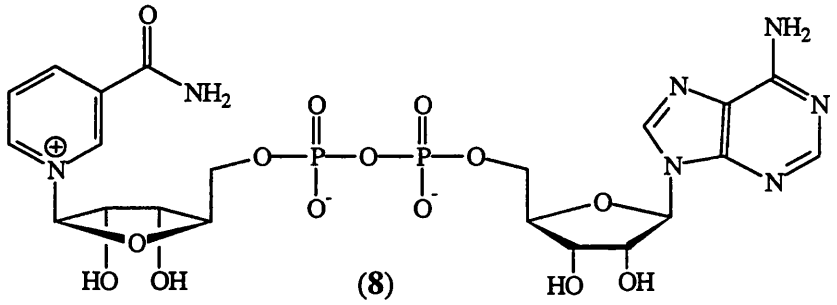
4.1 Introduction

The following chapter describes the synthesis of NAD^+ analogues by the coupling together of the synthetic adenosine 5′-monophosphates (Chapter 3) with NMN (**18**). It includes a review of the different methods available for this coupling and a discussion of the different compounds synthesised and the methods used.

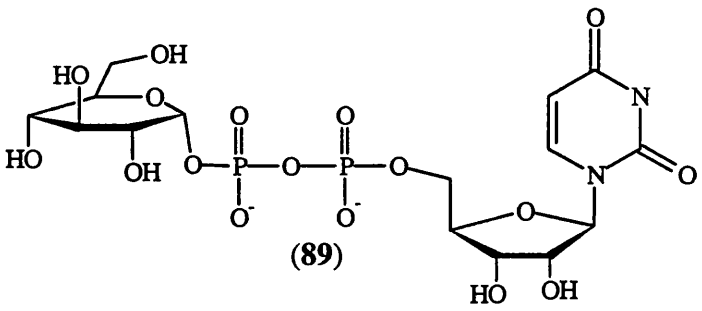
Nicotinamide adenine dinucleotide (NAD^+, **8**, section 1.9.2) is an example of a P^1, P^2-dialkyl ester of pyrophosphoric acid having one ester and a free hydroxyl group on each of the phosphate groups (figure 4.1). The pKa values of these two hydroxyls are similar to those of a monophosphate, i.e. approximately 1 and 6, and therefore, at physiological pH, at least one of these groups is ionised. This ensures the stability of the otherwise labile diphosphate ester against rapid hydrolysis. As discussed previously, NAD^+ is an important coenzyme for many different processes and it is thought that, in general, the pyrophosphate moiety acts as a structural unit providing coulombic binding to appropriate enzyme residues.[197] This is in contrast with another naturally occurring P^1, P^2-dialkyl pyrophosphate, that between uridine 5' -phosphate and hexose-1-α-phosphate (**89**, figure 4.1). In this compound it is the pyrophosphate linkage which is metabolically labile, making it a useful source of the active form of the glucose sugar residue.

In general, the chemistry of NAD^+ and its analogues is severely limited by the nature of the pyrophosphate bond. The compounds are highly soluble in water due to the ionised phosphate groups, the positive charge of the nicotinamide ring and the sugar hydroxyls and thus are insoluble in organic solvents without protection of these features. At ambient

pH and at room temperature the pyrophosphate link is stable to hydrolysis but will cleave when subjected to strong acid or base conditions or at elevated temperatures. It is for these reasons that the formation of this phosphate-phosphate linkage is the final step in chemical synthetic routes and several different methodologies have arisen for such syntheses.



(8)



(89)

Figure 4.1 Structure of NAD^+ (**8**) and UDP-Glucose (**89**).

Similarly to the monophosphates, ^{31}P NMR is a useful technique in the study of pyrophosphate species. When two symmetrical phosphates are bonded together the ^{31}P NMR exhibits a singlet with a chemical shift of approximately -10ppm. However, when in an unsymmetrical P^1, P^2-dialkylated pyrophosphate ester the two phosphorus atoms have slightly different chemical shifts and couple with each other to give two doublets between -11 and -12ppm, $J_{PP} \approx 15\text{-}20$Hz. These doublets are so close together in chemical

shift that the peaks become distorted, and an ABq is observed (figure 4.2). The problems of obtaining a phosphorus NMR spectrum, that were described previously (section 3.1) were further complicated for the NAD^+ analogues since there was often a very small amount of sample with which to obtain the spectrum.

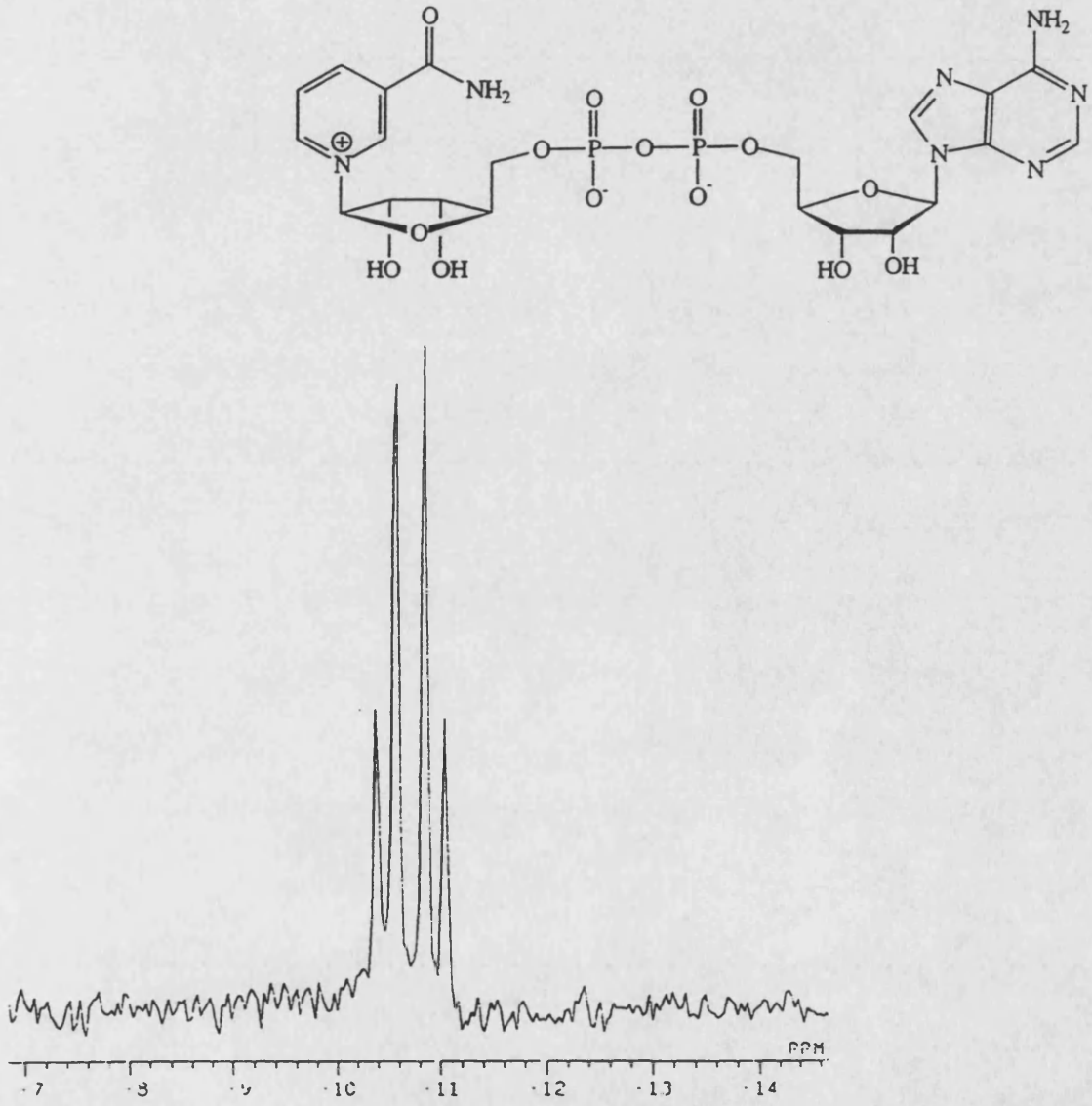


Figure 4.2 ^{31}P NMR Spectrum of an Unsymmetrical Pyrophosphate such as NAD^+ (**8**) (161.7MHz, solvent D_2O).

4.2 Methods of Synthesis of Nicotinamide Adenine Dinucleotide Analogues

Many analogues of NAD^+ (**8**), which acts as a cofactor in a wide range of biological reactions, have been synthesised in order to investigate its mechanism of action.[198] However, since it is the nicotinamide ring which is reduced in such biological processes, most alterations to date have been made in the nicotinamide mononucleotide half of the molecule. Early syntheses of these analogues used enzymatic methodology [199] modified from the biosynthesis, which involves the transfer of the adenyl unit from ATP to nicotinamide mononucleotide.[200] Later, several chemical syntheses were developed involving firstly the activation of a monophosphate by coupling to a good leaving group, and then reaction with a second phosphate (nucleophilic attack at the activated phosphate centre and displacement of the leaving group) to give the resultant pyrophosphate linkage (figure 4.3).

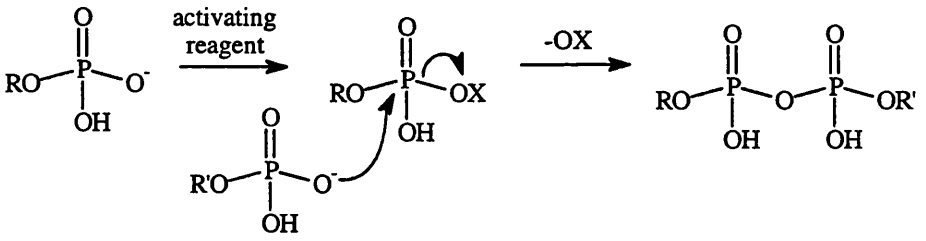


Figure 4.3 General Method of Formation of Pyrophosphate Linkage.

A brief overview of the major methodology for the synthesis of pyrophosphates is given below.

4.2.1 Phosphorochloridates

Pyrophosphate compounds can be thought of as analogous to the carbon based mixed acid anhydride chemical group (figure 4.4).

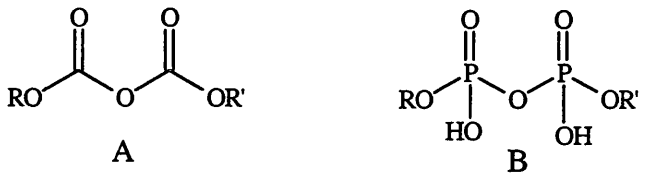


Figure 4.4 General Structures of a Mixed Acid Anhydride (A) and a Pyrophosphate (B).

A traditional method of preparing carbon mixed acid anhydrides is the reaction of the silver salt of one of the acids with the second acid activated as a chloride.[201] It was thought that a similar reaction utilising appropriately protected phosphate esters would lead to the formation of a pyrophosphate and this methodology was successfully applied to the synthesis of ADP (**21**).[202] Treating the silver salt of adenosine 5'-benzyl phosphate with dibenzyl phosphorochloridate, and subsequent removal of the benzyl groups, gave the product (figure 4.5). This was not only the first chemical synthesis of a pyrophosphate but also the first synthesis of a biological coenzyme.

Unfortunately, a weakness in this route, is that the tetraesters of pyrophosphates are particularly susceptible to a wide variety of exchange reactions by anionic attack. Hence, the yields of this reaction were considerably improved by treating the protected phosphorochloridate with the salt of a monoesterified phosphate to produce a triesterified protected intermediate, the so-called triester method. This species, which now had one

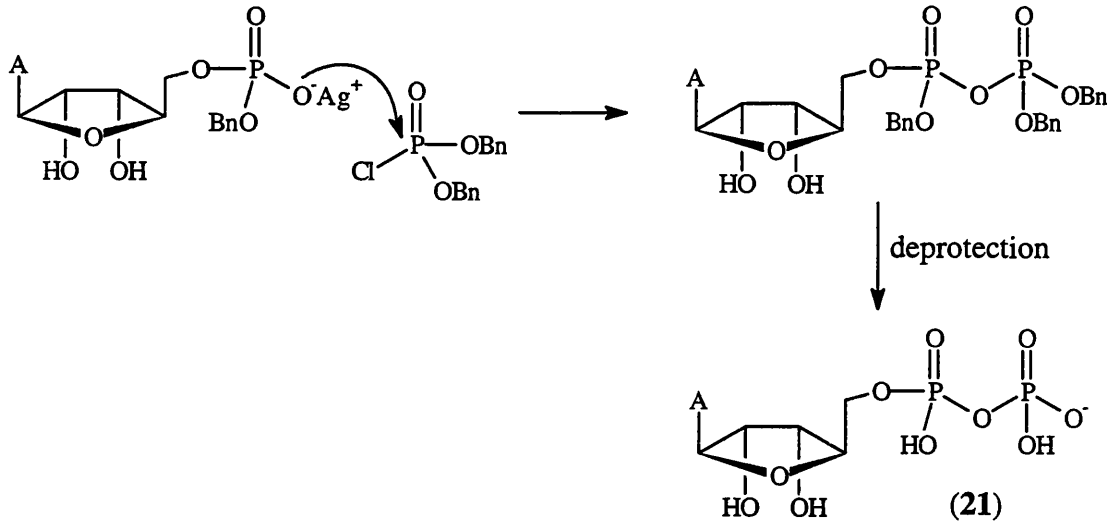


Figure 4.5 The First Synthesis of ADP (**21**).

ionised hydroxyl, was resistant to subsequent anionic attack. For example, when the tri-*n*-octylammonium salt of AMP (**19**) was reacted with dibenzyl phosphorochloridate and the product deprotected, ADP (**21**) was achieved in yields of 80%, compared to the 18% yield achieved via the tetraesterified intermediate.[203] This reaction was also used to successfully synthesise uridine diphosphate (**90**) and thymidine diphosphate (**91**) but due to the poor solubility of guanosine monophosphate (**92**) and cytidine monophosphate (**93**) in the reaction solvent (dioxane) these nucleotides did not react well.[204]

The triester method was similarly applied to the synthesis of the diesterified pyrophosphate coenzyme flavine adenine dinucleotide (**94**, figure 4.6).[205] 2', 3'-*O*-Isopropylidene adenosine-5' benzyl phosphorochloridate (**95**) was directly condensed with the mono silver salt of riboflavin-5' phosphate (**96**) in phenol. Unfortunately, following deprotection, the yield of the pure product from this first synthesis was very low (6%) but this was to some due extent to the harsh conditions used to remove the protecting groups causing hydrolysis of the pyrophosphate bond.

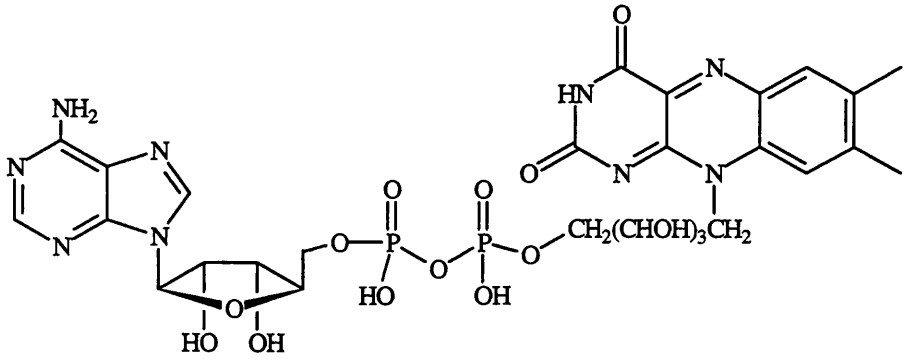


Figure 4.6 Structure of Flavine Adenine Dinucleotide (**94**).

It therefore became obvious that if this general route was to be extended to a more successful synthesis of asymmetric pyrophosphates, the choice of protecting groups for the phosphoryl hydroxyl was a critical problem. It was an extension of the monophosphate work which led to the introduction of phenyl groups to protect these phosphates. Thus, in a manner analogous to that outlined for the benzyl protecting group, diphenyl chlorophosphate was used successfully to synthesise a series of diphosphates. By the use of this method, the introduction of DMF as a co-solvent, and use of base catalysed hydrolysis to cleave the phenyl groups, guanosine diphosphate (**97**) and cytosine diphosphate (**98**) were prepared for the first time.[206]

Despite now being able to effectively synthesise polyphosphates, the high yielding synthesis of diesterified pyrophosphates, such as NAD^+, was still a challenge. Early attempts to use a second nucleotide to attack the phosphorus and cleave the P-O-P bond of a triester of pyrophosphoric acid failed due to the high stability of the intermediate in the reaction solvent dioxane.[204] However, following a study of the solvent effects on the reaction of esterified pyrophosphates, [207] it was found that, in the presence of pyridine, the intermediate could be attacked by a wide variety of anions of acids weaker than diphenyl phosphoric acid itself.[208] Since nucleotides are considerably weaker acids than diphenyl

phosphoric acid this method was able to be successfully used to give the first high yielding syntheses of a range of pyro- and polyphosphates including flavin adenine dinucleotide (**94**, 70-80%),[204] uridine diphosphate glucose (**89**, 62%) [209] and adenosine 5'-triphosphate (**22**, 77%).[207]

4.2.2 Other Mixed Anhydride Methods

Although phosphorochloridates and the triester method have been used extensively for the synthesis of pyrophosphates, other nucleotide anhydrides, for example those formed with TsOH or trifluroracetic acid have also been used [210] and indeed, one of the first syntheses of NAD^+ (**8**) utilised a trifluoroacetic acid mixed anhydride intermediate.[211] Trifluoroacetic anhydride was reacted with AMP (**19**) to give a mixed nucleotide anhydride which was then in turn attacked by NMN (**18**) to yield the product (18%). The major drawback of this method was that, due to the relative acidic strengths, the displaced trifluoroacetate anion was able to react with the newly formed pyrophosphate, cleaving the product, and reforming the mixed anhydride intermediate (figure 4.7).

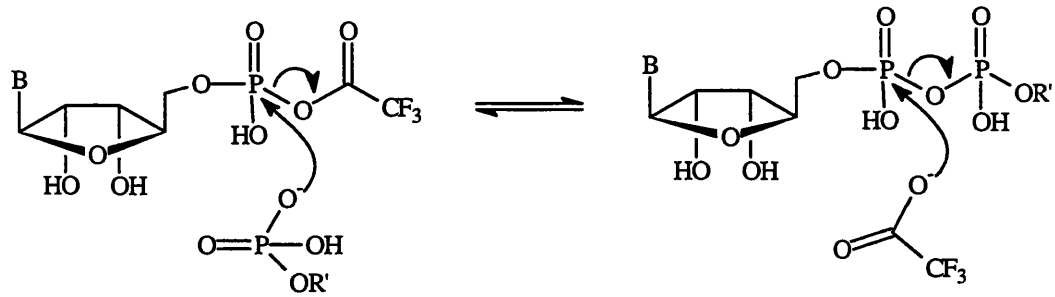


Figure 4.7 Mechanism for Trifluoroacetic Acid Mixed Anhydride Approach to the Synthesis of Pyrophosphates.

In general, these two mixed anhydride approaches (both the triester method and the trifluoroacetic acid procedure) to the synthesis of polyphosphates have many inherent advantages - namely the reaction can be controlled by activation of one phosphate followed by the addition of a second and if the correct leaving group is used then subsequent reaction can be prevented. However, there are also some disadvantages, including the necessity for anhydrous conditions to prevent the intermediate being cleaved by water, the requirement for protection and deprotection of the phosphorus groups and the difficulty of ensuring that highly hydrophilic compounds are soluble in organic solvent systems.

4.2.3 Carbodiimides

It was in order to overcome the problems of the mixed anhydride approach as outlined above that carbodiimides were introduced as an effective, if less elegant, means of coupling phosphate groups. The use of this coupling reagent to create pyrophosphate bonds obviated both the need for the protection of free acidic groups and the intermediate preparation of activated chloro-phosphates.

Carbodiimides are known to be effective dehydrating agents due to the highly unsaturated nature of the two double bonded nitrogen atoms, and their reaction in concentrated solutions with strong acids, such as the esters of phosphoric acid, is instantaneous and exothermic.[212] The most widely used carbodiimide for the dehydration of two phosphates to form a pyrophosphate has been N, N-dicyclohexyl carbodiimide (DCC) [213] but more recently a water soluble form of the reagent 1-ethyl-3-(3-dimethylaminopropyl)

carbodiimide (EDC) has been developed.[214] In general, the reaction proceeds with the first phosphate attacking the carbodiimide to form an intermediate with a good leaving group. Subsequently, nucleophilic attack by a second phosphate causes elimination of the hydrated carbodiimide, now a urea, and the formation of the pyrophosphate product (figure 4.8). When first developed this reaction was undertaken in anhydrous pyridine or DMF,[215] but it was later observed that it proceeded equally well in a water:pyridine mixture provided that a large excess of the carbodiimide was used.[216] This enabled the method to be utilised for the coupling of nucleotides which were insoluble in either DMF or pyridine, for example NMN, and hence an effective synthesis of NAD^+ (**8**) was developed.[217]

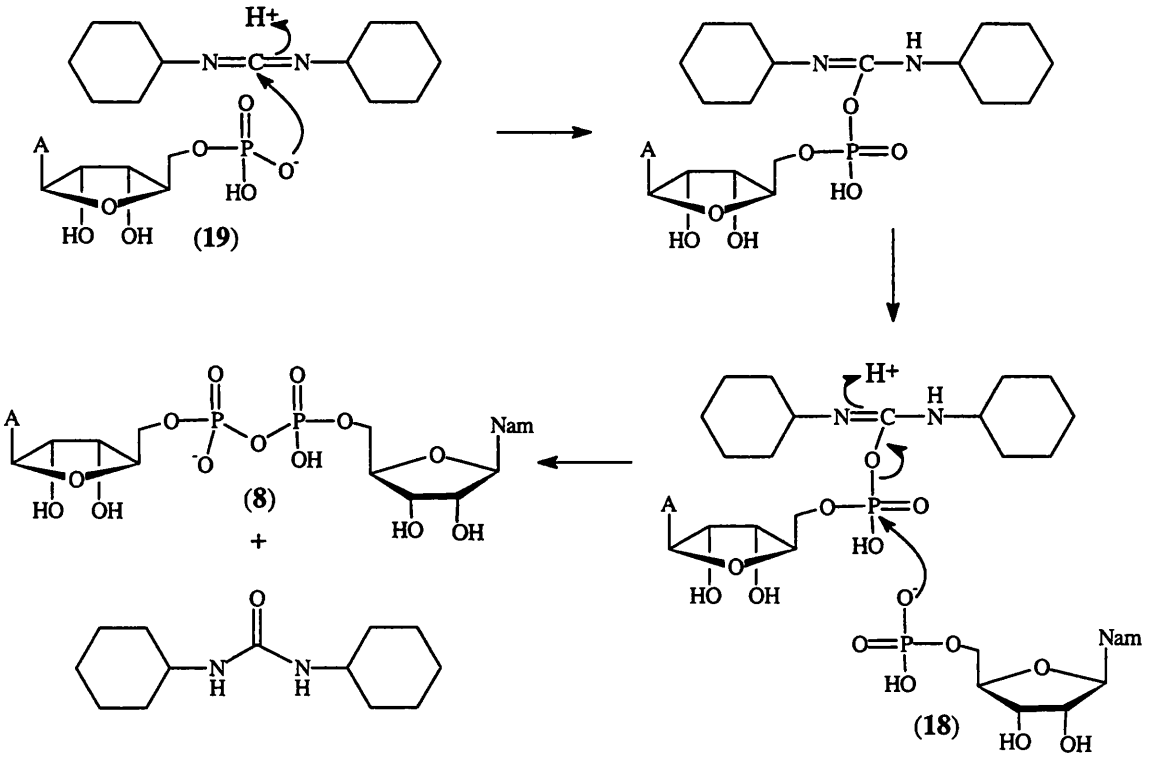


Figure 4.8 Proposed Mechanism of DCC Coupling Reaction for the Synthesis of NAD^+ (**8**) where A=adenine and N_{am}=nicotinamide.

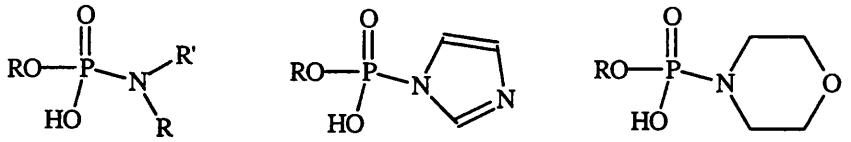
The major drawback to this methodology is that, when used to link pairs of acids with comparable nucleophilicity, there are three possible products, two symmetrical products and one asymmetric product. This both reduces the yield of the desired compound and produces a product mixture which needs to be separated carefully by ion-exchange chromatography. Secondly, this reaction is not suitable for the synthesis of polyphosphates using orthophosphoric acid since the multiple unprotected hydroxyls react rapidly and repeatedly giving a complex mixture of polyphosphate products.

However, one of the few asymmetric pyrophosphate systems which have been synthesised with a reasonable yield using this reaction is NAD^+, and its analogues. The reason for this success is the zwitterionic nature of NMN in the condensation medium. By analogy to argument proposed for the synthesis of CDP-choline,[218] the positive charge on the pyridine ring of NMN renders it a stronger acid than AMP, and therefore a poorer nucleophile for carbon. Thus, the initial attack on DCC is by AMP. However, since phosphoryl groups are good nucleophiles for phosphorus, the intermediate adduct can be attacked equally by either NMN or a second AMP molecule, to give either NAD^+ or diAMP respectively with little diNMN formed.[219]

4.2.4 Phosphoramidates Including Phosphormorpholidates

A final class of reagents which will react selectively with phosphoryl groups to afford polyphosphates are organophosphorus compounds containing a P-N bond. The phosphorus atom of the reagent is attacked by a nucleophilic phosphate with the

subsequent displacement of the nitrogen containing ligand. There are several reagents of this type [220] including the phosphoramidates, which exist in solution as zwitterions, and the phosphormorpholidates which are soluble in organic solvents (figure 4.9). These intermediates are relatively stable and can be isolated making them versatile and often the reagent of choice for the synthesis of pyrophosphate esters, especially those of nucleotides with similar acidity. The development of this methodology succeeded in the first synthesis of coenzyme A [221] and the synthesis of nucleoside diphosphate sugars.[222]



A B C

Figure 4.9 Structure of (A) a General Phosphoramidate and two specific examples (B) Phosphorimidazol and (C) Phosphormorpholidate.

To summarise, several routes for the synthesis of dialkyl pyrophosphates have been described which were, in general, developed in the late 1950's and early 1960's. However, little work has been performed more recently, and this literature, including comprehensive NMR data, is sparse. Of those papers which are available a complete mixture of routes has been adopted [223-225] with varying degrees of success and no one method has been highlighted as the most useful.

4.3 Synthesis and Purification of NAD^+ Analogues

The first preparation of NAD^+ analogues for subsequent enzymatic cyclisation to analogues of cADPR adopted the carbodiimide method of coupling and utilised the water soluble reagent EDC.[67] This method required the use of $MgCl_2$ counter ions, a ten fold excess of NMN, and buffering the reaction with HEPES at pH 6.8. Attempts to repeat this coupling by co-workers in this laboratory could not achieve the yields (40-50%) which Walseth and Lee had reported.

Therefore, we initially turned to the more traditional carbodiimide DCC and this was used successfully to couple several commercial monophosphates with yields of approximately 20-30%.[97] A large excess of DCC was added to a mixture of 5:8 NMN : AMP (or analogue) dissolved in 1:4 water : pyridine and stirred at room temperature for seven days.[217] The reaction was quenched by addition of excess water and the DCU by-product was allowed to settle by standing at low temperature. This solid was then conveniently removed by filtration, and the NAD^+ analogue was purified by ion exchange chromatography.

A major problem with this method arose, however, when attempts were made to use it to couple synthetic AMP analogues to NMN. During the long reaction time (7 days) any inorganic phosphate, introduced to the reaction as a contaminant of the synthetic AMP material, was able to couple readily to the nucleotide forming unwanted polyphosphate products. Thus, although this method was very simple to execute, it could only be used to couple those synthetic monophosphates which were seen to be pure of inorganic

phosphate by ^{31}P NMR. A second limitation of this method was that it could not be used to couple nucleotides which had been altered in the phosphate moiety and it was for these reasons that a second coupling method was sought.

A thorough search of the literature revealed that the diphenyl chlorophosphate (DPPC) mixed anhydride method of activating monophosphates for coupling had been used successfully in recent years to synthesise NAD^+ analogues.[226] Activation of the nucleotide was by reaction with DPPC, and attack of a second nucleotide then displaced the acidic diphenyl. In theory either NMN (**18**) or AMP (**19**) could be initially activated by DPPC (there was literature precedent for both procedures [227-228]) and, although both of these routes were used successfully to synthesise the parent compound, better yields were consistently achieved by the selective activation of the AMP nucleotide (10% against 24%). A second problem which this reaction route presented was that NMN could not be induced to dissolve in the reaction medium, DMF. This was overcome by the initial protection of the NMN 2'- and 3'- hydroxyl as acetate esters and deprotection of the NAD^+ product by methanolic ammonia.

A typical procedure for this coupling reaction was as follows (figure 4.10). 2', 3'-Di-*O*-acetyl NMN (**99**) was synthesised by reaction of NMN (**18**) with acetic acid in pyridine.[227] After removal of the solvent, any phosphate anhydride was cleaved by reaction with pyridine/water and the material was used directly in the next step. Simultaneously the anhydrous tri-*n*-octylammonium salt of AMP (**19**) was activated by reaction with DPPC (1.5 equivalents) in anhydrous dioxane and DMF. After reaction, excess DPPC was removed by triturating in dry ether. Activated AMP (**100**) was then taken up in 1:1

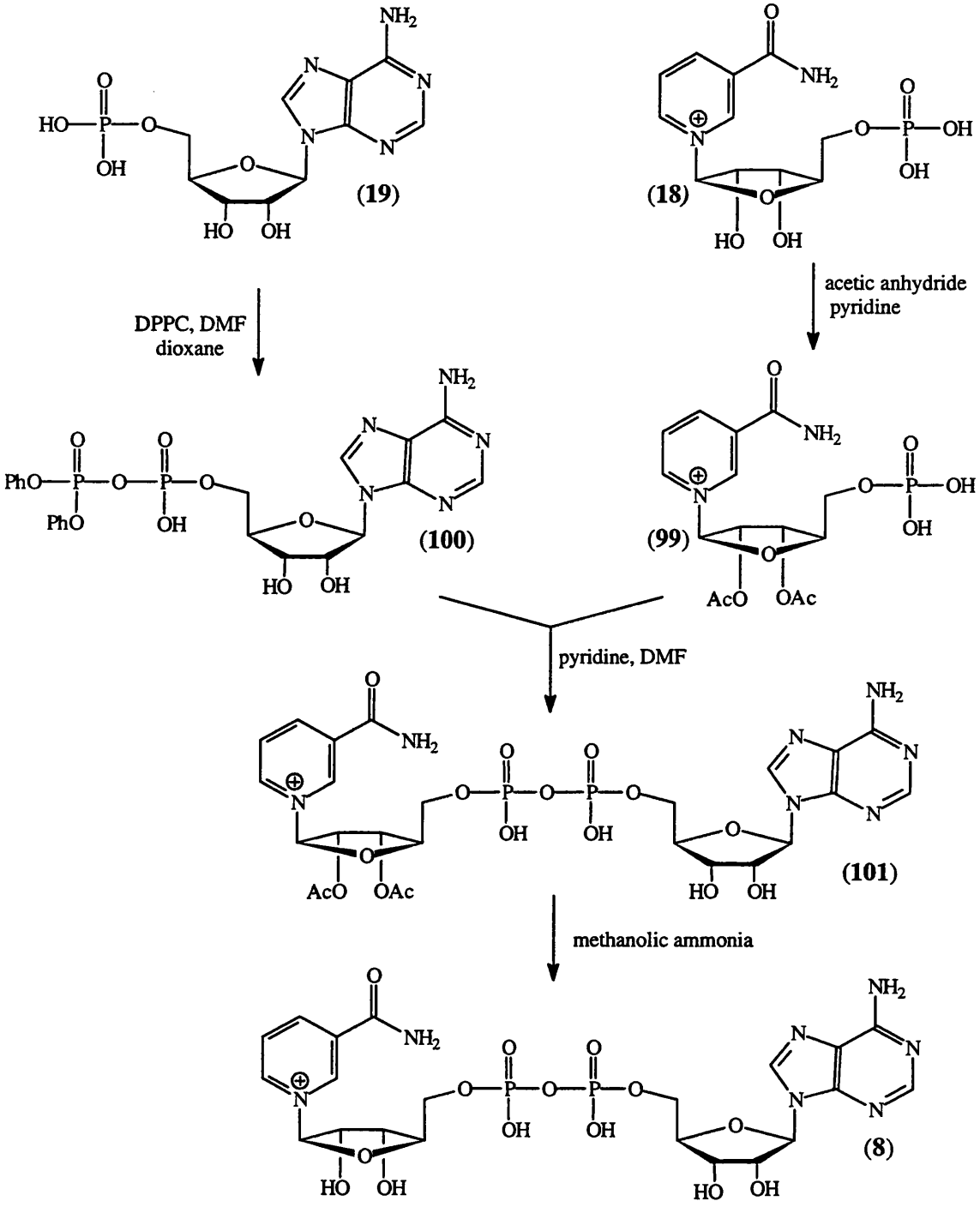


Figure 4.10 Synthesis of NAD^+ and Analogues Using Diphenyl Chlorophosphate Method.

pyridine:DMF solution, added to the tri-*n*-octylammonium salt of 2', 3'-di-*O*-acetyl NMN (**99**) and stirred overnight. Following removal of the solvent, the acetates were removed

from the protected pyrophosphate (**101**) by reaction with 50% methanolic ammonia at 0°C and the product (**8**) purified by ion-exchange chromatography.

The formation of the activated pyrophosphate intermediate was followed by NMR. Initially DPPC (1.1 equivalent) was added in one portion to a solution of the tri-*n*-octylammonium salt of AMP dissolved in a mixture of dioxane and DMF. After 2h at room temperature ^{31}P NMR showed there to be no monophosphate remaining but disappointingly, there were several peaks in the NMR spectrum: a singlet at -12.25ppm, two doublets at -13.7ppm and -23.8ppm and a second singlet at -25.78ppm (figure 4.11). The two doublets corresponded to the required activated pyrophosphate intermediate and the singlet at -25.78ppm was unreacted DPPC, which was removed from the reaction mixture by treatment with cold dry ether. Further investigation indicated that the peak at -12.25ppm was bisAMP (**102**) which presumably formed by the side reaction of any unreacted AMP (**19**) with the activated AMP intermediate (**100**). Attempts to suppress this side reaction by addition of DPPC dropwise and cooling the reaction mixture before and during the addition had little effect. The best result was achieved by addition of a 1.5-2 fold equivalents of DPPC, although this was still unable to prevent the formation of bisAMP completely. Addition of a greater excess of DPPC than this provided no further reduction in the amount of bisAMP (**102**) formed.

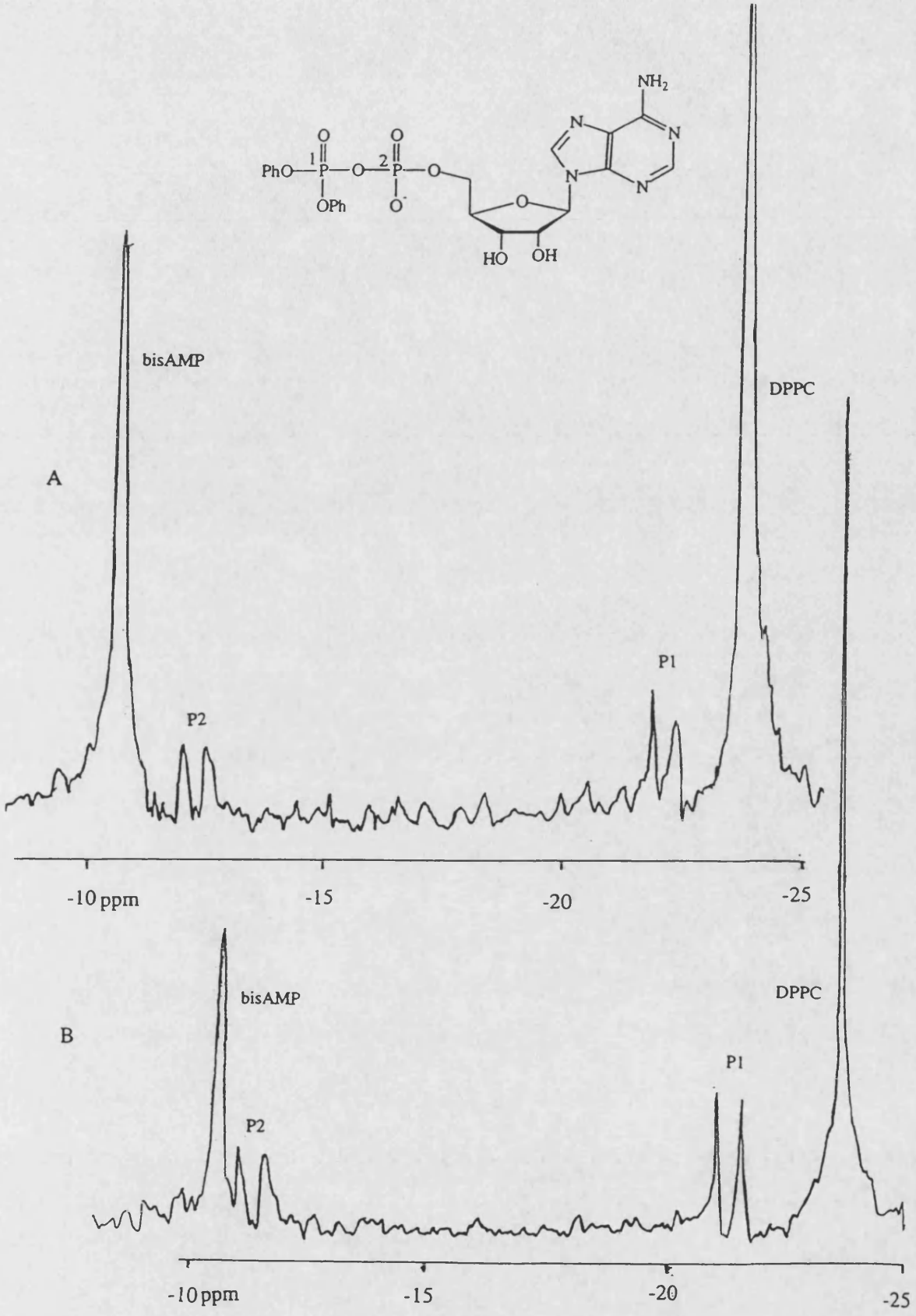


Figure 4.11 33MHz ^{31}P NMR Spectrum of the Formation of the Activated DPPC Coupling Intermediate. (A) Shows the Spectrum when DPPC (1.1equivs) was used and (B) the Reduction in the By-Product BisAMP Achieved with the Use of DPPC (1.5-2equivs).

Although the published method for the alkaline hydrolysis of the NMN hydroxyl esters required reaction for 6 hours, further work in this area indicated that these esters were in fact very base labile. In an attempt to isolate $2'_N$, $3'_N$-di-*O*-acetyl NAD^+ (**101**) the crude product from the coupling step was loaded directly on to the ion exchange column. Investigation of the fractions by HPLC showed the esterified product to be present, but, after removal of the buffer *in vacuo*, only NAD^+ itself could be isolated. Hence, as the water had evaporated the rise in pH of the solution to approximately pH=11 made the medium basic enough to cause the rapid hydrolysis of the esters *in situ*, resulting in the desired compound being lost. To prevent this happening the ion exchange eluant was adjusted to pH=6 using 1M HCl before concentration and the pH was monitored throughout removal of the solvent.

A summary of all the NAD^+ analogues synthesised in this project is shown (table 4.1).

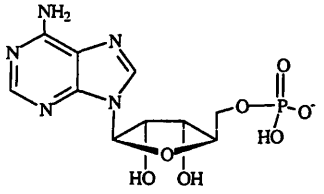
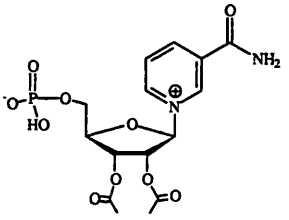
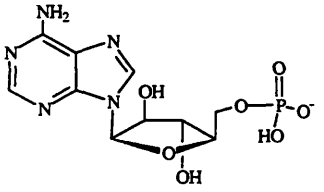
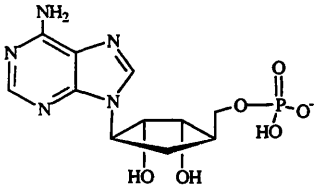
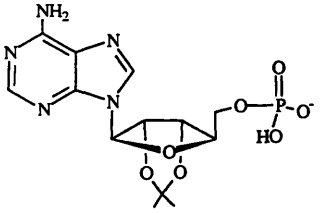
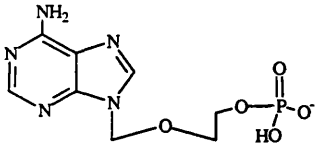
AMP replacement phosphate	NMN replacement phosphate	Compound	Method of Coupling	Yield
	NMN	**8** NAD+	both DPPC and DCC	24% and 20%
AMP		**101** AcONAD+	DPPC	8.7%
	NMN	**103** NAraD+	DCC	10%
	NMN	**104** NArisD+	DPPC	19%
	NMN	**105** NAcetD+	DCC	14.3 %
	NMN	**106** NAcycloD+	DCC	12%

Table 4.1 Summary of Coupling Reactions.

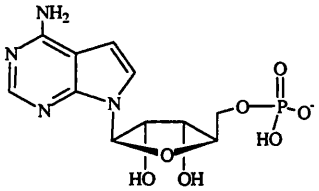
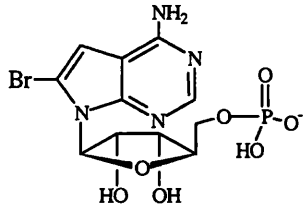
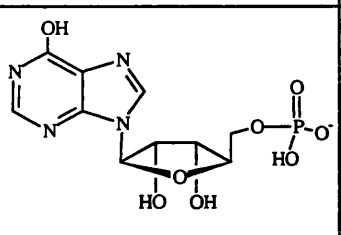
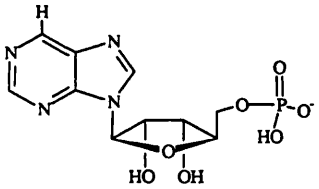
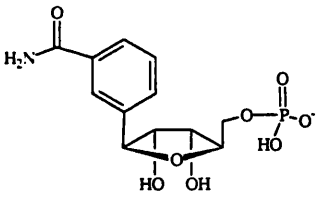
AMP replacement phosphate	NMN replacement phosphate	Compound	Method of Coupling	Yield
	NMN	**107** 7-deaza-NAD^+	DPPC	38%
	NMN	**108** 7-deaza-8-bromo-NAD^+	DCC	27.7%
	NMN	**109** NID^+	DPPC	27%
	NMN	**110** NPD^+	DCC	75%
	NMN	**111** NBD^+	DCC	12.4%

Table 4.1 Summary of Coupling Reactions (Continued).

A section of the ^{1}H NMR spectrum of 7-deaza-8-bromo-NAD$^+$ (**108**) is shown (figure 4.12) illustrating the aromatic protons and the two anomeric positions and is an example of what was more generally observed. An unusual feature of this NMR is that the H_A2' proton is shifted downfield as a result of the bromine atom forcing the adenine ring into a *syn* conformation (section 2.5). The only other NAD$^+$ analogue in which ribose ring protons, other than the anomeric protons, were observed downfield of the water peak was the 2', 3'-*O*-isopropylidene analogue (**105**).

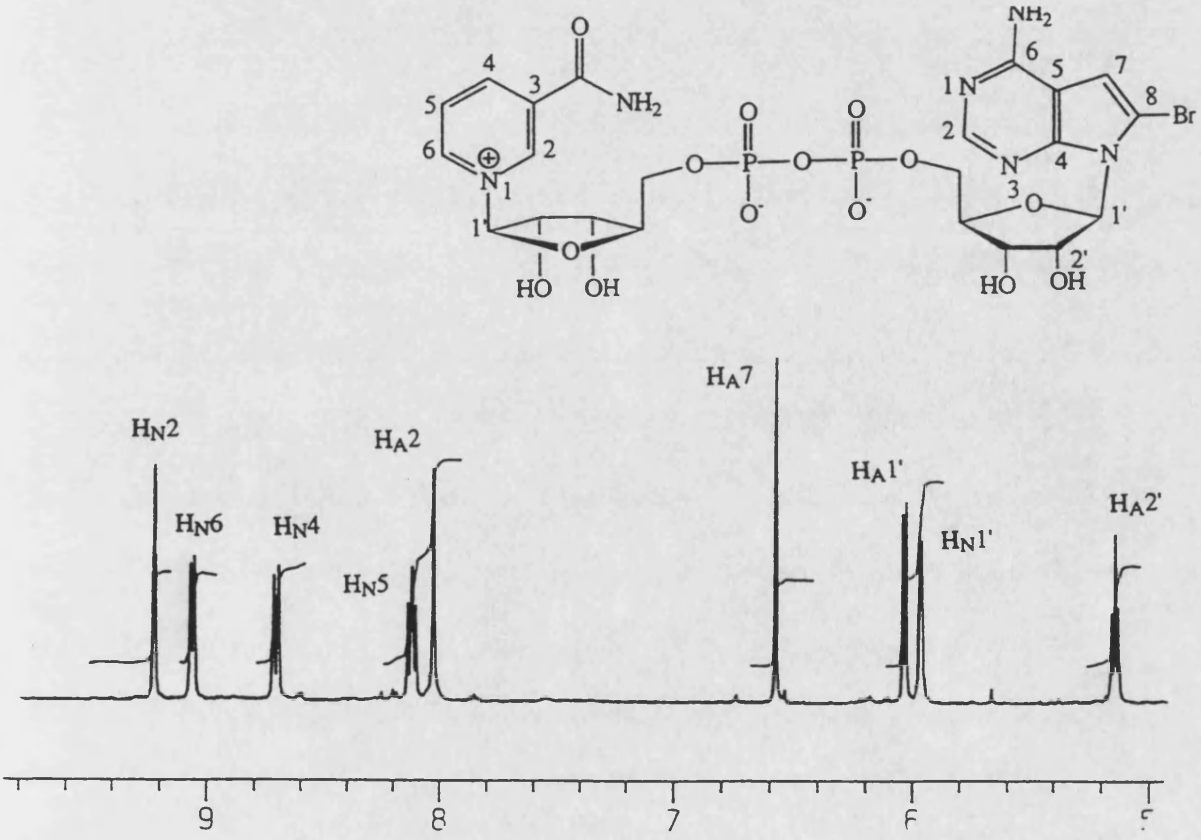
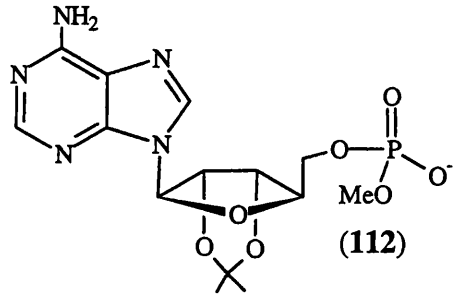


Figure 4.12 Section of the ^{1}H NMR Spectrum of 7-deaza-8-bromo-NAD$^+$ (**108**) Showing the Protons Down-field of 4.8ppm (400MHz, solvent D_2O).

It is of interest to note that when the 2', 3'-*O*-isopropylidene analogue was synthesised a second product was isolated. This was a monophosphate which ^{1}H NMR revealed to be

2', 3'-*O*-isopropylidene adenosine 5'-(O-methoxy)monophosphate (**112**, figure 4.13) and which had arisen from the coupling of the AMP analogue (**76**) to residual methanol in the sample. It appeared that, in the case of the isopropylidene analogue, residual MeOH in the monophosphate, which had been used to remove excess triethylamine from the product after elution from the ion exchange column, had not been effectively evaporated and had coupled to the starting material.



(**112**)

Figure 4.13 By-Product from the 2', 3'-*O*-Isopropylidene Coupling Reaction.

4.4 Attempted Combined Phosphorylation and Coupling Reaction

Since all the methods discussed for the formation of pyrophosphate intermediates involve the activation of the phosphate with a good leaving group, it was postulated that it might be possible to directly form NAD^+ analogues by addition of NMN to the dichlorophosphate phosphorylation intermediate without prior isolation of the monophosphate product. No literature precedent for this type of reaction exists to the best of the author's knowledge.

To a solution of 2', 3'-*O*-isopropylidene adenosine (**42**, 0.16mmol) in triethylphosphate (0.6ml) was added, after cooling, $POCl_3$ (0.65mmol). After 45min the solution was

evaporated *in vacuo* and to this added 2', 3'-di-*O*-acetyl NMN (**99**) tri-*n*-octylammonium salt (0.5mmol) in DMF (3ml) and pyridine (0.5ml). The reaction was stirred at room temperature overnight and then the acetates removed using 50% methanolic ammonia (10ml) at 0°C. The crude product mixture was purified by ion exchange chromatography to give a major peak which eluted between 70 and 90mM TEAB. ^{31}P NMR (figure 4.14) identified this to be the AMP adduct with which the NMN had reacted twice but, since HPLC showed the material to be impure, the reaction was taken no further. A second smaller peak eluted from the ion exchange column between 150 and 180mM TEAB but the ^{31}P consisted of a complex mixture of ABqs at -11ppm and -23ppm and therefore it was more difficult to propose a possible structure for this material. However, this procedure does show some potential, and with development, it might be possible to combine the troublesome phosphorylation and coupling reactions into one step.

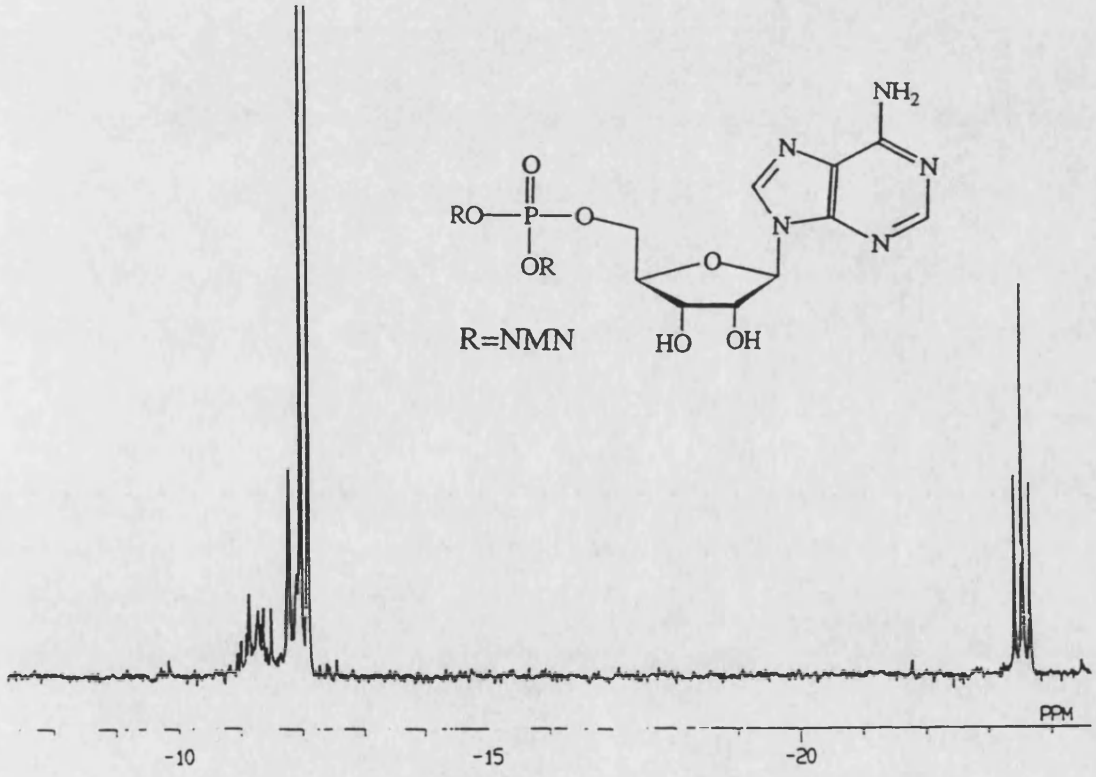


Figure 4.14 ^{31}P NMR Spectrum of the Product from the Attempted Phosphorylation and Coupling Combined Step (161.7MHz, solvent D_2O).

CHAPTER FIVE

ENZYMATIC SYNTHESIS OF CYCLIC ADENOSINE 5'-DIPHOSPHATE RIBOSE ANALOGUES

5.1 Introduction

This final chemical discussion chapter deals with the final step in the synthesis of cADPR and its analogues, the enzymatic cyclisation of β-NAD^+ (**8**), or analogues of β-NAD^+, to give the target compounds. As previously, this chapter begins with a review of the different methods of synthesis of cADPR and then discusses the analogues synthesised in this project.

As discussed (section 1.9) a synthetically useful total chemical route to cADPR (**7**), or its analogues, does not yet exist and thus the only synthesis available for generating such compounds is enzymatic cyclisation of the biological precursor β-NAD^+ (**8**). This involves cyclisation of the dinucleotide with loss of nicotinamide to create a new bond, that between the N1 of the adenine ring and the C1' of the nicotinamide ribosyl. In the natural diastereoisomer this bond is formed with retention of stereochemistry at the C1' centre, namely the nicotinamide ribosyl linkage and the N1 cyclic ribosyl linkage are both β with respect to the ribosyl ring.[228] Therefore, the new bond is not formed simply by a concerted nucleophilic attack of the adenine N1 at the C1' anomeric centre with the subsequent displacement of nicotinamide (figure 5.1A). Instead, the new link must be formed with inversion which arises from the nicotinamide group leaving before attack of the nitrogen occurs from the same side of the ribose ring (figure 5.1B).

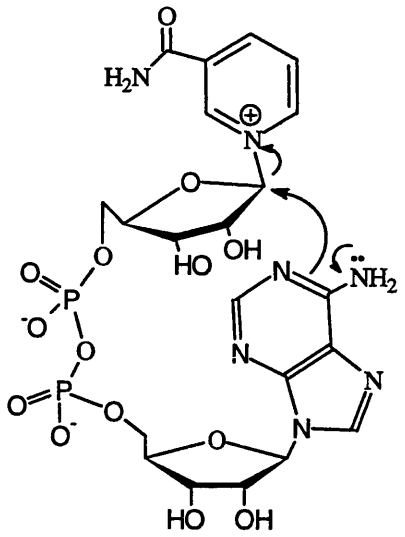


Figure 5.1A Schematic Concerted Cyclisation of NAD^+ (**8**).

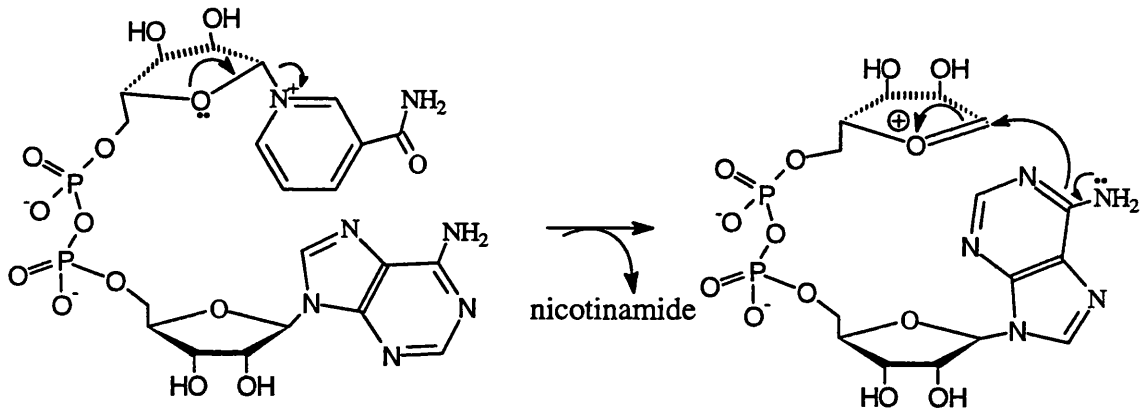


Figure 5.1B Cyclisation of β-NAD^+ (**8**) to give cADPR (**7**) Occurs with Retention of Stereochemistry.

Chemical macro-cyclisation of molecules remains a challenging problem since any such closure requires a reduction in entropy which must be overcome by an increase in the free energy. Therefore, for large rings the greater entropy reduction necessary is a reflection of the relative improbability of achieving the required molecular orientation. To be successful, macrocyclisations must not only be performed in dilute solutions to avoid

polymerisation, but often also need to be carried out at elevated temperatures to overcome the necessary energy barrier, eg the cyclisation of ω-bromoalkyl malonate.[229] A further implication of large molecules needing to orientate themselves correctly for such cyclisations is that rate of reaction can become very slow.

When considering the particular cyclisation of NAD^+ (**8**) there are a number of inherent problems. Firstly, NAD^+ (**8**) is highly hydrophilic but also susceptible to hydrolysis, so prolonged heating of a solution of NAD^+ (**8**) could cause cleavage of the pyrophosphate bond, but to dissolve NAD^+ in other solvents requires a high degree of protection combined with the use of a dipolar solvent. The cyclisation of NAD^+ also requires stereochemical and conformational restraints to be overcome by correct orientation of the flexible pyrophosphate linkage. Even if selective cleavage of the ribosyl nicotinamide bond could be achieved then cyclisation could still proceed by attack of several different purine nitrogens or from either of the faces of the ribosyl sugar ring.

Despite these difficulties Yamada *et al.* [230] have reported a nonenzymatic cyclisation of β-NAD^+ (**8**) to give the correct stereoisomer of cADPR (**7**). The method involves heating β-NAD^+ (**8**) in anhydrous DMSO with various metal halides, the best yield being achieved with NaBr. The halide displaces the pyridinium ring with inversion of stereochemistry, and the N1 of the adenine displaces the halide to give the cyclised product with the correct stereochemistry. After purification of the product the yield was 11.5% and the reported yields for analogues of NAD^+ ranged from 5.6-16.8%. Additionally, the reaction was carried out on a relatively large scale (approximately 50mg of NAD^+) and with a low yield

this method was not readily adaptable to the small quantities of synthetic NAD^+ analogues which were products from the previous steps.

The use of enzymes in organic synthesis is increasing in popularity [231] and for stereoselective synthesis enzymatic catalysis can be ideal producing consistently only one diasteroisomeric product. Enzymatic cyclisation of β-NAD^+ (**8**), using the natural metabolic enzyme ADP-ribosyl cyclase (section 1.5.1), not only results in a stereospecific reaction, but is also a technique that can readily be adapted to small scale work. However, the inherent disadvantage of relying upon enzymes in synthetic work is that any analogue of the natural substrate may not itself be bound by the enzyme active site in the same manner, which can lead to different products or no reaction at all. However, in this case, *Aplysia* ADP-ribosyl cyclase appears to have a very loose substrate specificity and various different alterations of the natural substrate have been well tolerated.[97]

Other enzymatic routes have also been reported for the synthesis of small amounts of cADPR. Some NADases, enzymes which are readily available from mammalian tissue, are also able to catalyse the cyclisation of β-NAD^+ to cADPR. The specific activity of these enzymes from most tissues is too small to be of synthetic use but, it is reported that such enzymes isolated from pig brain can be used successfully to cyclise small amounts of β-NAD^+ (**8**) to cADPR (**7**) (1.5-4% yield).[228]

The only other reported route to cADPR has used a NAD^+ pyrophosphorylase enzyme to catalyse the cyclisation of N1-(5'-phosphoribosyl)ATP (figure 5.2).[228] The reaction, which was performed in a mixed organic-aqueous buffer, gave cADPR in low yield (7%). It was

discovered that it was essential to use an organic buffer mix since in a purely aqueous solution the enzyme intermediate complex was hydrolysed so quickly that cyclisation did not occur and N1-(5'-phosphoribosyl)AMP was the only product isolated.

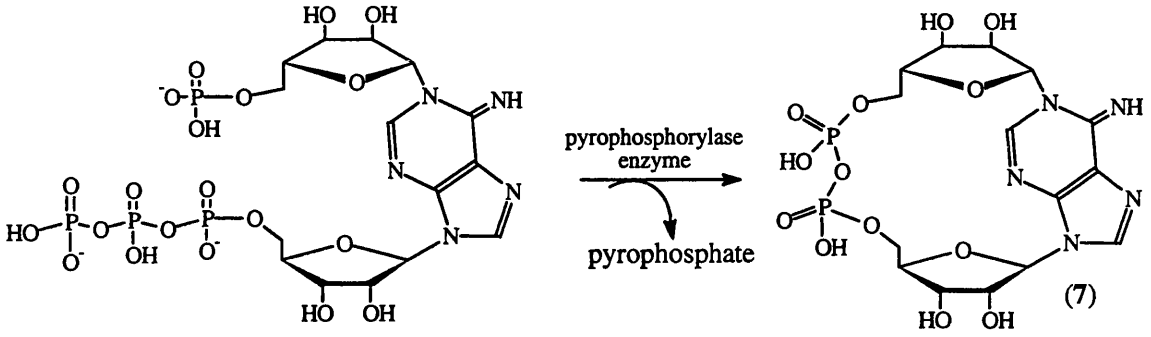


Figure 5.2 Synthesis of cAPDR Using a NAD^+ Pyrophosphorylase Enzyme.

Hence, the only viable route to cADPR analogues at present is to cyclise analogues of β-NAD^+ (**8**) using the natural enzyme. A subtype of this enzyme with particularly high cyclase activity has been purified from the sea slug *Aplysia californica*.[32] Despite not being commercially available until last year, this group was fortunate in that a large supply of enzyme, which had been isolated crude from *Aplysia* and could be stored at -70°C with only a small loss of activity, was kindly donated by our colleagues in Oxford. Therefore, following the chemical synthesis of β-NAD^+ analogues, the final cyclisation step was carried out using this enzyme preparation. This method not only provides a useful synthesis of cADPR analogues, but can also be used to probe the structure activity relationship of the cyclase with its natural substrate. Any analogues of NAD^+ which were not cyclised could be further tested for inhibition of the enzyme.

5.2 Enzymatic Synthesis of Analogues of cADPR

In general, the cyclisation method used was that reported by Ashamu *et al.*.[97] An appropriate amount of the triethylammonium salt of the β-NAD^+ (**8**), or an analogue, was dissolved in 25mM HEPES solution at pH 6.8 to give 2.5ml of a 1.5mM solution. To this was added the crude enzyme (10μl), the reaction mixture shaken, and the cyclisation allowed to proceed at room temperature. The enzyme was pH sensitive and was deactivated by an even mildly acidic pH, and hence careful buffering of the reaction medium was important. The cyclisation was monitored by HPLC using an increase in the peak size corresponding to the by-product nicotinamide as a guide as to whether or not the reaction was complete (figure 5.3). When the area of this peak was no longer seen to be increasing, normally approximately 40min, the cyclisation was quenched by dilution with MilliQ water and the mixture was purified immediately using ion exchange chromatography.

The cyclic product was generally susceptible to hydrolysis, being converted to its ADPR analogue, (cADPR has a half life of 24h in solution at room temperature [33]) and was therefore treated with appropriate care. The final compounds were stored at -70°C to prevent degradation.

Novel analogues of cADPR altered in the ribose moieties were quantified by UV using the published extinction coefficient of cADPR ($14.3 \times 10^3 M^{-1}cm^{-1}$).[27] However, when altered in the adenine ring, the novel compounds were quantified using a modification of Briggs' total phosphate analysis test.[233] A known aliquot of the product was destroyed by

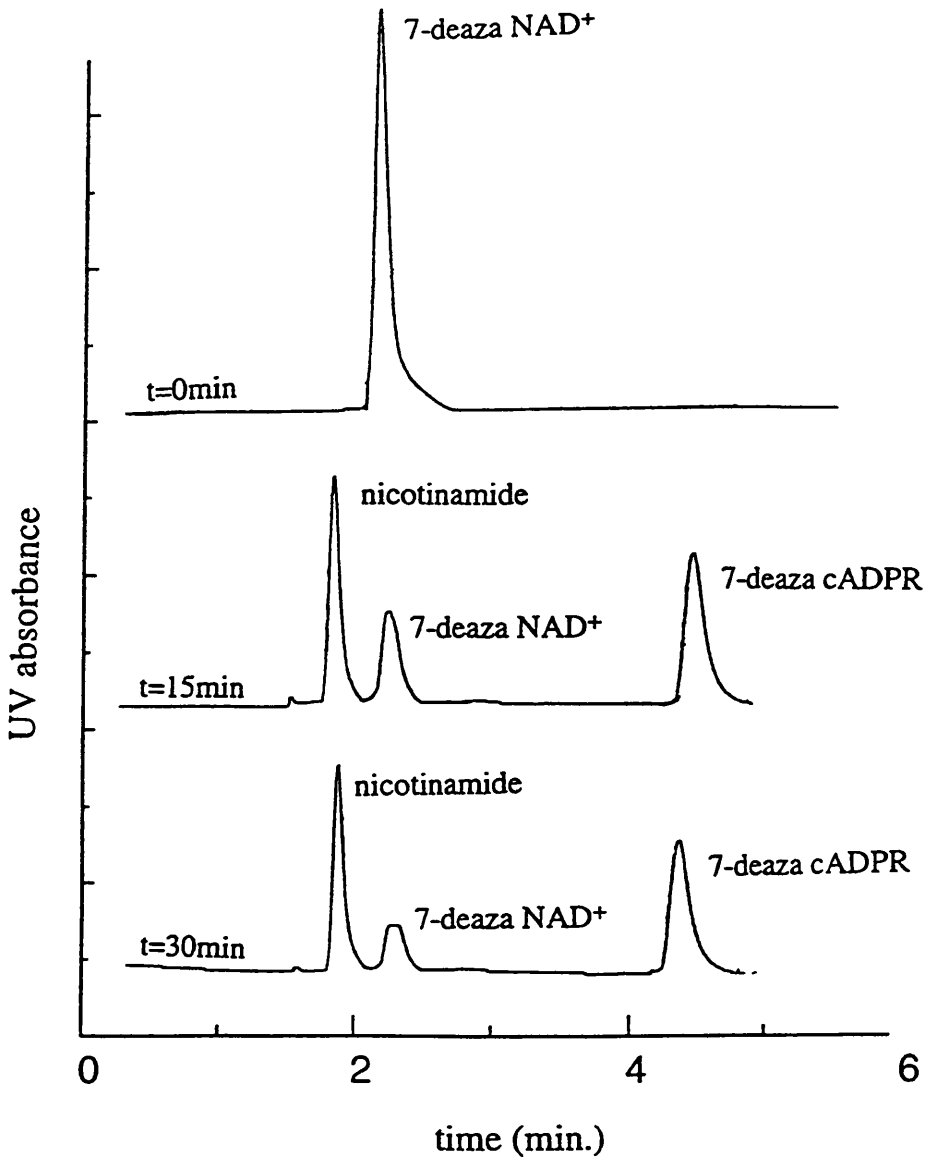


Figure 5.3 HPLC Trace Showing a Typical Cyclisation Experimental Time Course (SAX column, 0.05M KH_2PO_4 / 5%MeOH buffer).

heating with concentrated acid and then redissolved in an accurate volume of various solutions containing sulphite, molybdate and quinol. When heated this mixture formed a coloured complex with the inorganic phosphate and the UV absorbance at 340nm of this solution was a measure of the amount of phosphate present. Each reading was repeated in triplicate. The values were compared to a standard curve produced from accurately measured quantities of inorganic phosphate treated as above and prepared directly before

use. By accurately calculating the amount of phosphate in the solution it was possible to calculate the extinction coefficient of the product and this was used to quantify the analogue for all future use.

There were several spectral characteristics of the cyclic products which were useful in helping to characterise the them. The mass peak of the cyclic compound occurred at exactly 122 mass units less than for the corresponding NAD^+ analogue indicating the loss of nicotinamide. The UV spectrum of the cyclic material, if cyclised at the N1 position, had a maximum at a similar wavelength to that of the linear form. The HPLC retention time of the product, using our anion exchange system, was longer than for the precursor when cyclised at N1 (figure 5.3). The ^{31}P ^{1}H decoupled NMR spectrum showed an ABq at approximately -11ppm which was similar to that observed for the NAD^+ analogues (figure 5.4) and the ^{1}H NMR spectrum had several features of interest - the aromatic region now only contained the peaks arising from the adenine ring with those for the nicotinamide having been eliminated; there were two anomeric protons at about 6ppm both with a similar coupling constant; due to the angle that the adenine ring now adopted in relation to the adenosine ribose (approaching a *syn* conformation) the H_A2' proton was shifted downfield and now appeared at approximately 5.5ppm (figure 5.5).[234]

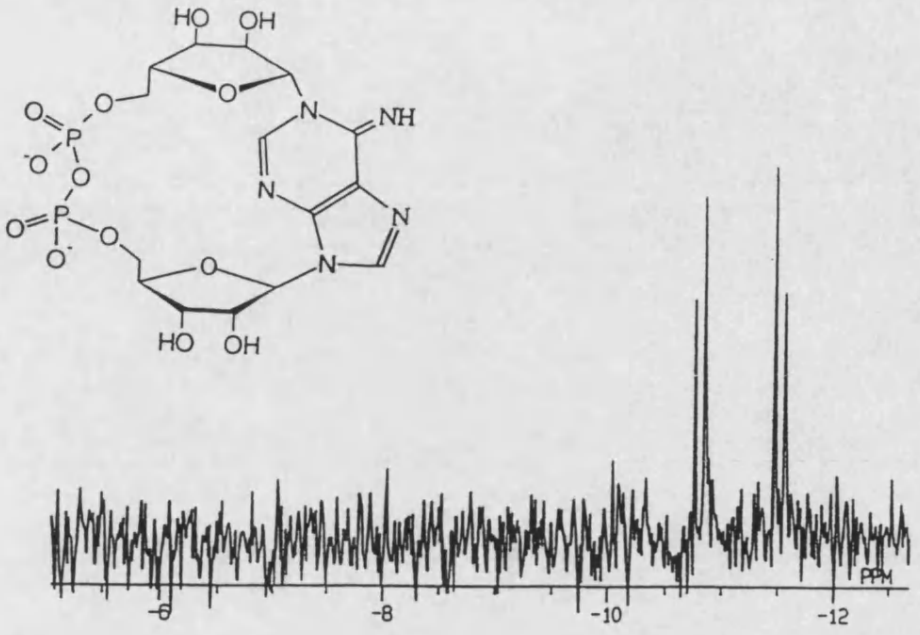


Figure 5.4 ^{31}P NMR Spectrum of a Typical N1 Cyclised Product such as cADPR (**7**) (161.7MHz, solvent D_2O).

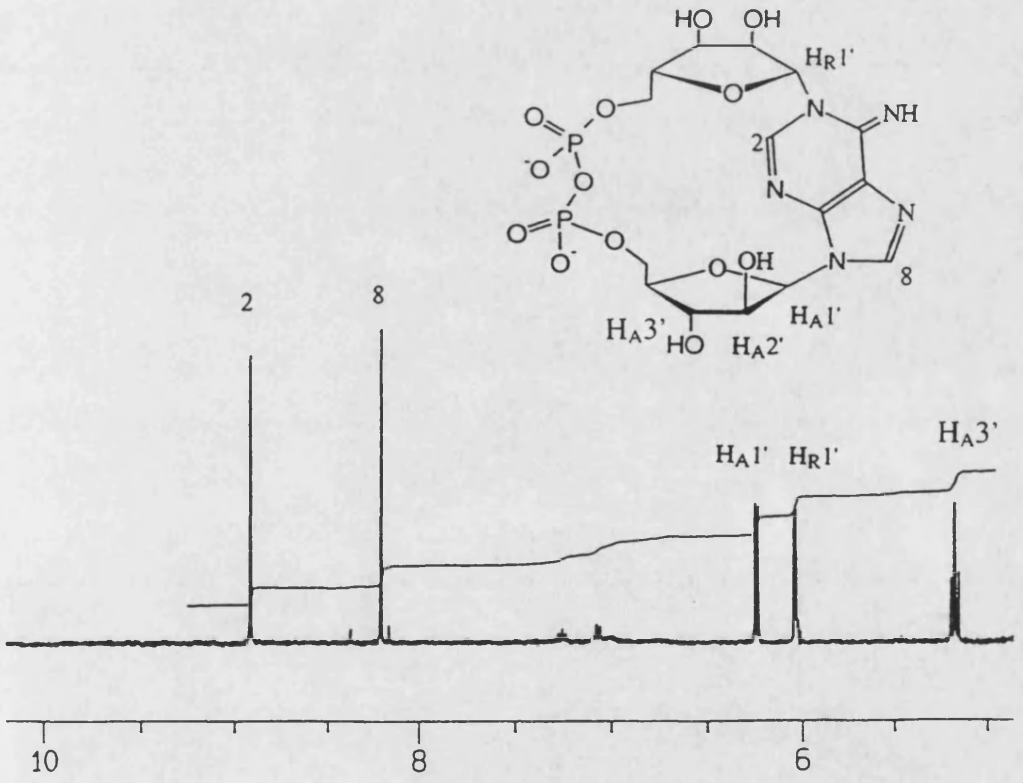
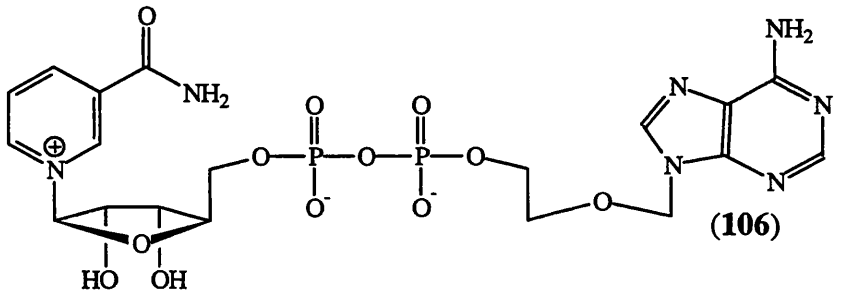


Figure 5.5 ^{1}H NMR Spectrum of cAraDPR (**113**) Showing the Peaks Down-field of 4.8ppm (400MHz, solvent D_2O).

5.2.1 Products Cyclised at the N1 Position

In the main the NAD^+ analogues were turned over by the enzyme with a qualitatively similar efficiency to the turnover of the parent compound to yield the N1 cyclised product. The products which were successfully synthesised via this route are shown (table 5.1).

The K_m for an enzymatic reaction is defined as the concentration of substrate at which the velocity of enzyme action is half the maximum velocity of that process. However, this value is more realistically used as a measure of the affinity of a particular substrate for the enzyme active site. For this particular cyclisation reaction the K_m for only one of the novel substrates was measured, that of NAcycloD$^+$, (**106**, figure 5.6).



(**106**)

Figure 5.6 Structure of NAcycloD$^+$ (**106**).

This value was of interest since NAcycloD$^+$ (**106**) has a far more flexible molecular structure than any other substrate the enzyme had been challenged with previously. When measured, the K_m of *Aplysia* ADP-ribosyl cyclase for the substrate NAcycloD$^+$ (**106**) was found to be 135μM, a similar order to that for NAD^+ (**8**) itself, calculated to be 100μM. Although it was known that the adenosine hydroxyl groups were not essential for enzyme - substrate binding (the enzyme is known to cyclise many analogues modified about this

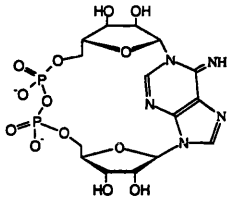
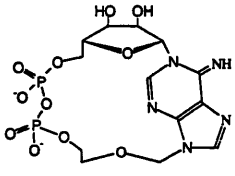
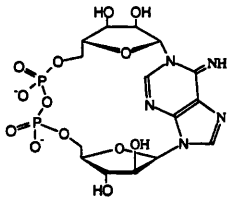
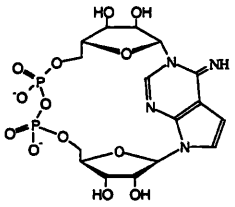
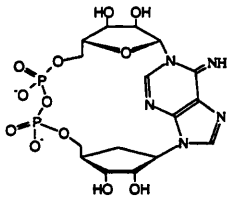
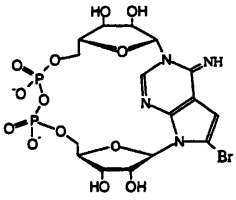
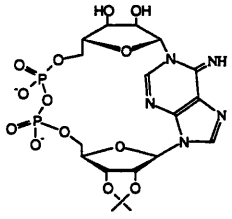
Structure	Compound	Yield	Structure	Compound	Yield
	cADPR **7**	56%		cAcycloDPR **116**	21%
	cAraDPR **113**	54%		7-deaza-cADPR **117**	57%
	cArisDPR **114**	71%		7-deaza-8-bromo-cADPR **118**	31%
	cAcetDPR **115**	46%	-	-	-

Table 5.1 Summary of N1 Cyclised cADPR Analogues.

position among them NAcetD$^+$ (**105**), NAraD$^+$ (**103**), 2'-deoxy NAD$^+$ (**119**) and 3'-deoxy NAD$^+$ (**120**) - that the ribose ring can be replaced by an alkyl chain with little or no loss of the enzyme's ability for substrate manipulation is of importance in the design of small molecule mimics of cADPR.

However, an investigation into the importance of the "northern" ribose for enzyme substrate binding gave very different results. When the enzyme was challenged with AcONAD$^+$ (**101**, figure 5.7), an analogue of NAD$^+$ in which the hydroxyl groups of the NMN ribose were protected with acetate groups, no cyclisation product was observed.

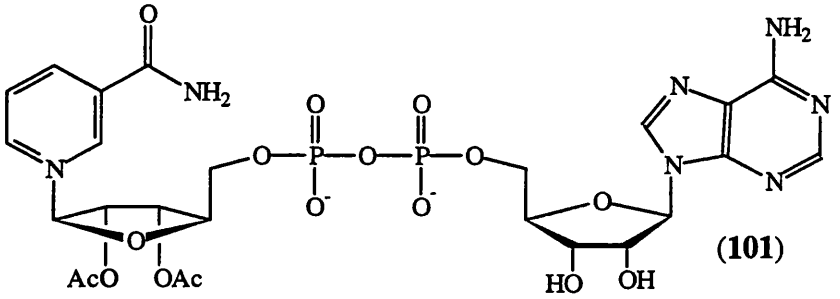


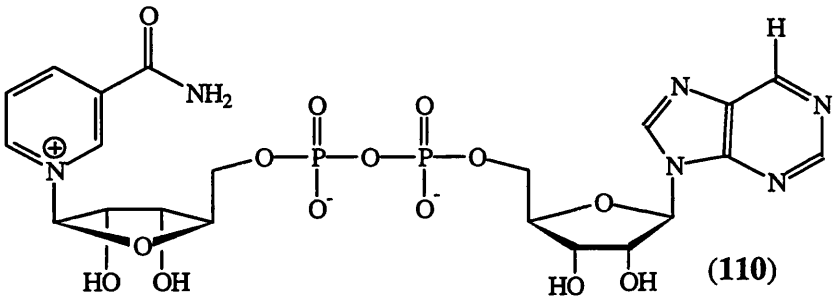
Figure 5.7 Structure of AcONAD$^+$ (**101**).

This compound was tested for its ability to inhibit enzyme-mediated cyclisation of β-NAD$^+$. A solution of both NAD$^+$ (100μM) and AcONAD$^+$ (100μM) in HEPES was incubated with the enzyme and the reduction in the rate of cyclisation was compared to a control experiment. AcONAD$^+$ caused only a 19% reduction in the amount of nicotinamide released when compared to the control, and therefore only a small amount of inhibition in the rate of cyclisation of NAD$^+$. This result indicates that this modified substrate candidate is only poorly recognised by the enzyme active site and, as a result,

only minimally prevents the competitive binding and cyclisation of the natural substrate NAD^+. It thus appears that the "northern" ribose hydroxyls are essential for binding the analogue into the active site such that the enzyme can accept the substrate for cyclisation.

When analogues of adenosine are substituted at the 8-position of the purine ring the large molecular volume of the substituent prevents free rotation about the ribose purine ring and forces the purine from the more usual *anti* conformation into a *syn* conformation (section 2.5). The ring is now in the correct conformation to enable N1 cyclisation to occur readily and this might explain why large substituents at the 8-position (such as bromine and piperidine), which would normally be expected to disrupt a delicate enzyme substrate binding interaction, are tolerated in for these compounds. The additional knowledge that 7-deaza analogues of NAD^+ (**107**, **108**) are cyclised readily adds further evidence that the imidazol ring is unimportant for cyclase-substrate interaction.

The effect of a modification of the N6 position of the adenine ring upon cyclisation of these substrates was also studied. NPD^+ (**110**, figure 5.8) was synthesised, an analogue of NAD^+ in which the N6 amino group had been replaced by a simple H atom.



(110)

Figure 5.8 Structure of NPD^+ (**110**).

The attempted cyclisation of this analogue appeared initially to proceed in the usual fashion with HPLC data showing an increase in the nicotinamide peak, a decrease in the starting material peak and the appearance of a new peak at approximately 5 minutes. Disappointingly though, when the reaction was quenched HPLC evaluation showed that the material at 5 minutes had degraded to give a new peak at 9 minutes. Despite several attempts using buffers at different pH values to quench the reaction this initial product could not be stabilised. It therefore appeared that the compound had cyclised but due to the lack of any means of delocalising the positive charge, the cyclic compound rapidly degraded to an ADP-ribose linear analogue (figure 5.9). Unfortunately, due to the small amount of material available no further work could be performed on this reaction mechanism and no products could be isolated.

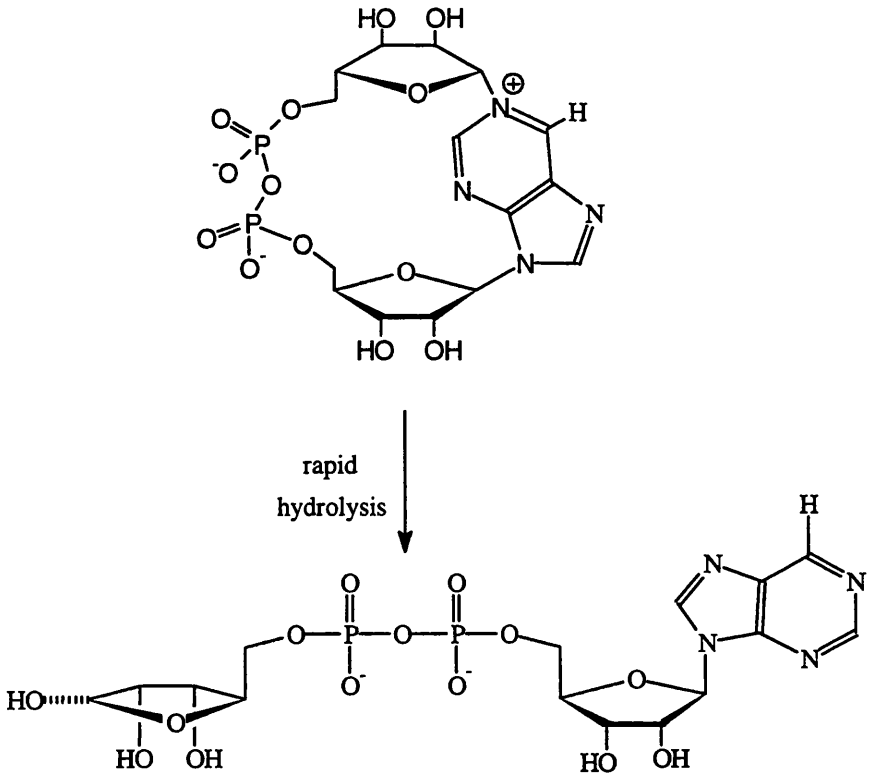
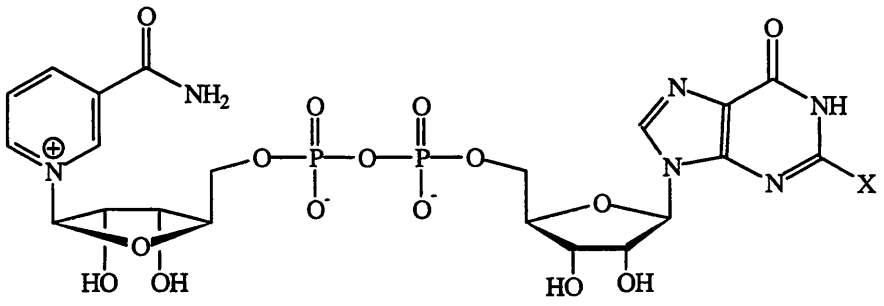


Figure 5.9 Proposed Rapid Hydrolysis of cPDPR Results in PDPR.

It is known that the N1 position of purine is more acidic than in adenine (pKa values are 2.5 and 4.1 respectively) and the same trend would be expected to be true of the NAD^+ analogues. Although little is known about the mechanism of hydrolysis of cADPR (**7**) and its analogues, the transition state probably involves neighbouring group participation of a ribosyl oxygen lone pair to displace the purine ring forming a carbocation which is the captured by water. The ability of the ribosyl linkage to be hydrolysed should therefore be dependent upon the leaving group ability of the purine ring. In this case the ring is so acidic that the cyclic material is spontaneously hydrolysed.

5.2.2 Products Cyclised at the N7 Position

In order to further investigate the role of the N6 amino group upon cyclisation of these analogues and upon the Ca^{2+} releasing ability of cADPR a series of target compounds were designed in which the NH_2 group was replaced by a hydroxyl (see figure 5.10 for tautomer).



NID+ (**109**), X=H NGD+ (**121**), X=NH_2

Figure 5.10 Structures of NID^+ (**109**) and NGD^+ (**121**).

Enzyme-mediated cyclisation of these NAD^+ analogues exhibited a number of differences from previous cyclisations. The cyclisation took much longer to proceed to completion than that for cADPR (approximately 2hours as opposed to 40minutes), required a 5 fold increase in the concentration of enzyme and the HPLC retention time of the cyclic product was similar to that of the NAD^+ analogue and approximately 1min faster, in our system, than that observed for cADPR. Since our HPLC system utilises an anion exchange column this implied that the product had a lower net charge than cADPR. Additionally purification of the cyclic products also provided some difficulties. Although a distinct product was observed at the low pH of the HPLC system, the material could not be purified by ion exchange chromatography using TEAB buffer. When monitored by UV no material was seen to elute from the column even at 100% buffer concentrations. This problem was overcome by alteration of this purification method and the use of an acidic buffer system to purify and isolate the product.

Once pure, the new analogues also showed several differences in their spectral characteristics when compared to cADPR. Most striking was the ^1H NMR of cIDPR and cGDPR which showed no distinctive downfield shift of the H2' proton implying that the purine ring did not lie in the *syn* conformation across the ribose ring (figure 5.11) and implying that the compound had not cyclised at the N1 position. A possible alternative position for cyclisation was at the N7 atom (figure 5.12).

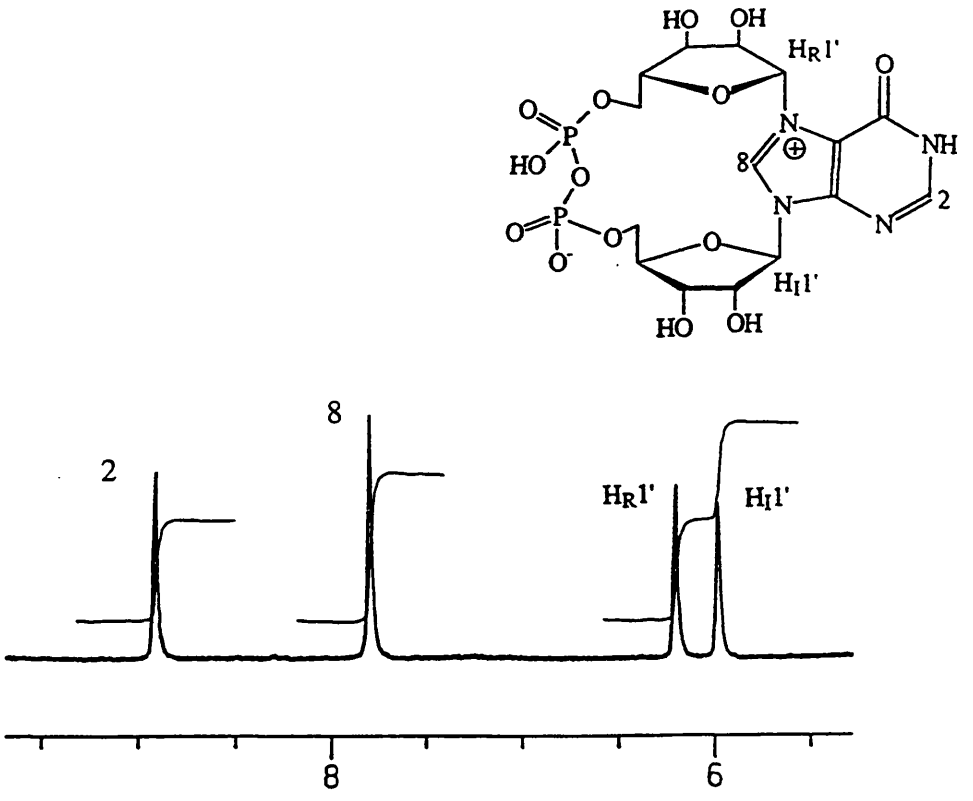
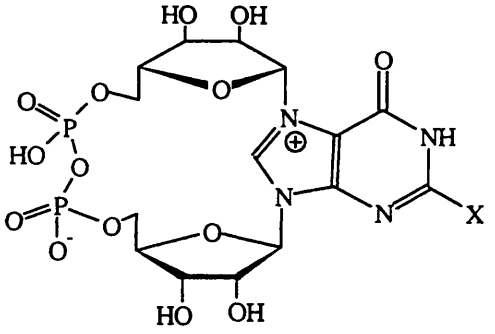


Figure 5.11 ^{1}H NMR of cIDPR (**122**) Showing Peaks Down-field of 4.8ppm (400MHz, solvent D_2O).



cIDPR (**122**), X=H
cGDPR (**124**), X=NH_2

Figure 5.12 Structure of N7 cyclised cIDPR (**122**) and cGDPR (**123**).

It is known that analogues of guanosine and inosine substituted at the N7 atom are fluorescent [236] and when investigated the cyclic compounds were also shown to be fluorescent (figure 5.13). This further implied N7 cyclisation.

Models of these N7 cyclised compounds showed them to be very much more flexible than their N1 cyclised counterparts and the presence of a positive charge upon the purine ring leads to the possibility of intramolecular ion pairing between the purine ring positive charge and one of the ionised phosphates. The separation of compounds by ion exchange chromatography requires the binding of the material to the anion exchange resin by an electrostatic interaction and then, by a combination of an increase in ionic strength and protonation of the phosphate residue, the product is eluted from the column. In the usual case there are two free phosphates and the pH of the solution needs only to be just below neutral to protonate one residue and cause the compounds to be eluted. However, as a result of the intramolecular ion pairing for compounds cyclised at N7, a dramatic reduction in pH, to values of approximately 1, is therefore required to protonate the remaining phosphate residue and elute the product from the column. It was for this reason that the TEAB buffer system could not be used to purify the product and a strongly acidic system was required.

Shortly after this work had been completed Graeff *et al.* [237] published cGDPR and confirmed our results of cyclisation at the N7 position. There followed several other articles on this work. One of these such papers reported the extinction coefficients at λ_{max} of cGDPR (**123**) and cIDPR (**122**) to be 13000 and 1000 $M^{-1}cm^{-1}$ respectively (at 257nm).[238] Although little experimental detail was recorded it appeared that these values were calculated directly from the mass of the cyclic material.

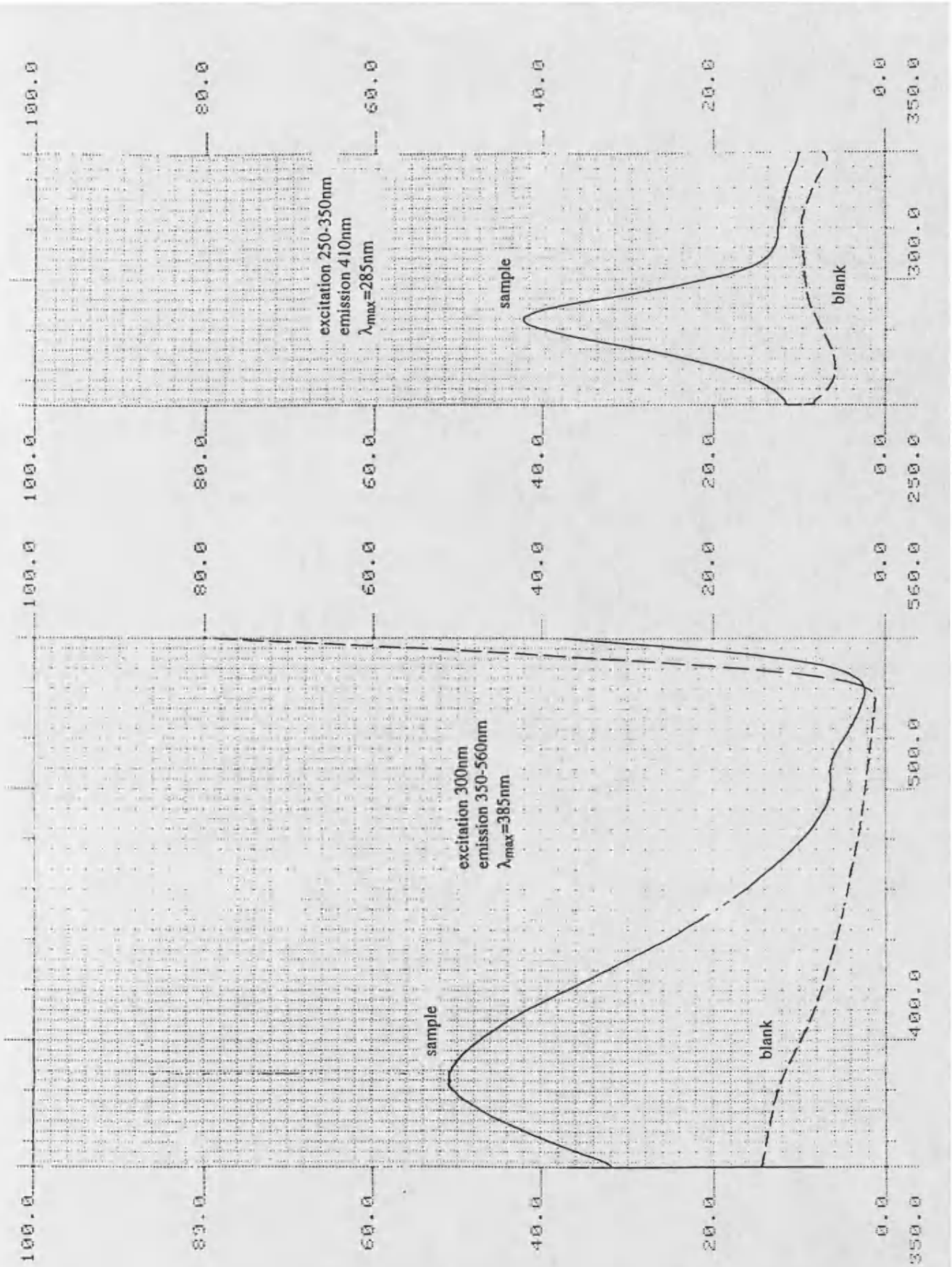


Figure 5.13 Fluorescent Traces of cIDPR (**122**). Similar traces were also obtained with cGDPR (**123**).

It is known that such compounds exist as salts with an unknown number of equivalents of counter ion and such calculations based upon mass alone are unreliable. When compared to our extinction coefficient values for cGDPR and cIDPR, calculated using total phosphate analysis, (8760 and 5000$M^{-1}cm^{-1}$ respectively, also at 257nm), it can be seen that a large discrepancy exists.

Zhang and Sih [238] also reported chemical cyclisation of such compounds using NaBr and DMSO, a method which had previously been successfully to cyclise NAD^+ (**8**) to cADPR (**7**).[230] However, this method also resulted in a compound which was cyclised at the N7 position. It would therefore appear that the position of cyclisation was not dependent upon the enzyme active site but instead upon the reactivity of the N1 position. The presence of the keto group in NGD^+ (**121**) and NID^+ (**109**) reduces the nucleophilicity of N1 such that formation of the cyclising linkage can only form with the N7 atom.

These results lead to some conclusions as to the mode of action and active site of the cyclising enzyme. It appears that the first step is the release of the nicotinamide group and the formation of an intermediate involving ADPR in which the anomeric carbon may well be in an activated state poised to cyclise. It also appears that within this complex the base is able to rotate freely around the N9-C1′ bond until the most reactive centre is aligned for cyclisation. Reaction then occurs and the product is released. This proposed mechanism is consistent with the enzyme not requiring base recognition for reaction which has been verified with cyclisation of many base modified analogues. This theory of reactivity may also explain the slower rate of formation of cGDPR (**123**) and cIDPR (**122**). Although in

the keto compounds the N7 is more reactive than N1 it can be postulated that this nitrogen is still a lot less reactive than N1 of adenine and hence cyclisation proceeds more slowly.

If this hypothesis is true it would appear that a keto compound in which the N9-C1′ rotation was restricted or in which the N7 atom had been eliminated would be an ideal inhibitor for this enzyme active site. Two compounds which could be used for this purpose are 8-bromo-NID$^+$ (**124**) and 7-deaza-NID$^+$ (**125**) (figure 5.14).

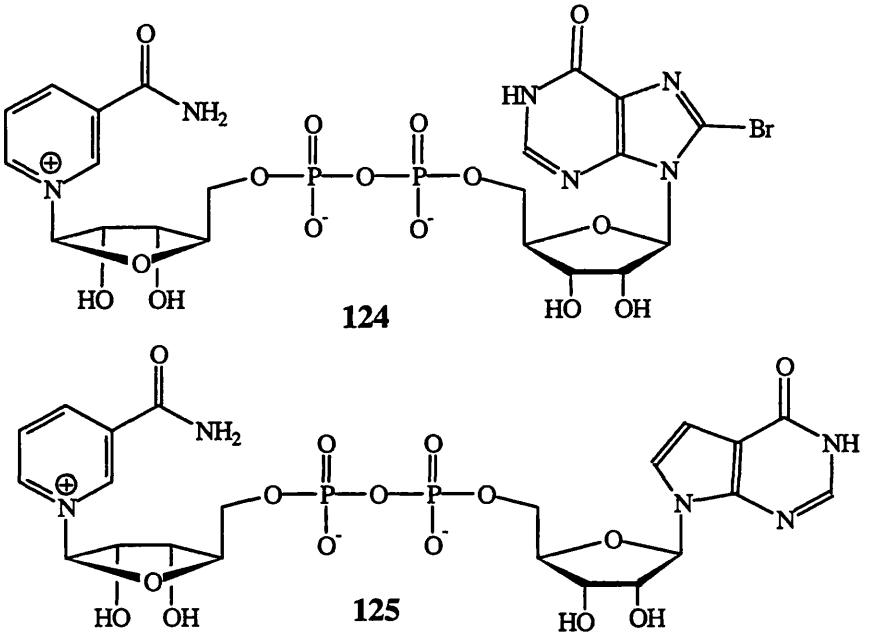


Figure 5.14 Structure of 8-Bromo-NID$^+$ (**124**) and 7-deaza-NID$^+$ (**125**).

5.3 Inhibitors of ADP-Ribosyl Cyclase

In addition to the inhibitor approach hypothesised above, a second approach for investigating possible inhibitors for ADP-ribosyl cyclase enzyme was to design a compound which was still able to mimic NAD$^+$ (**8**) accurately enough to fit into the active site but which would not react further. Thus it was decided to maintain the two ribose

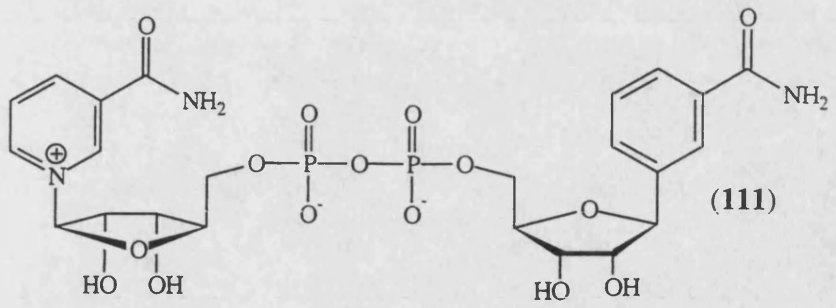
rings and relative stereochemistry, and to concentrate upon the aromatic moieties of NAD^+. It is known and understood that the adenine ring plays little or no part in the recognition site so interest was focused at the NMN half of the molecule. If it is thought that the reaction proceeds by initial elimination of nicotinamide to produce an activated enzyme-substrate complex which then cyclises there are two obvious approaches to designing an inhibitor.

Firstly, the purine ring can be removed so that there is no possible moiety to cyclise and the activated enzyme substrate complex does not proceed to cyclisation. A possible flaw with this approach is that the activated enzyme-substrate complex could be hydrolysed to give the free enzyme and an "ADP-ribose" like linear product.

A second approach to designing such an inhibitor is to synthesise a substrate which closely mimics the nicotinamide moiety of NAD^+ but does not have the same leaving group ability ie which will fit into the enzyme active site but will not form the enzyme substrate active complex. A good candidate for this would be replacement of the nicotinamide ring with a benzamide ring.[239] One drawback of this approach might be that the substrate would lack an electronegative positive charge which could be instrumental in correctly locating NAD^+ in the ADP-ribosyl cyclase active site.

Since there were possible flaws in both of these approaches a first step inhibitor was designed which combined both ideas, nicotinamide benzamide dinucleotide (**111**, figure 5.15). This NAD^+ analogue was synthesised by coupling NMN (**18**) with benzamide mononucleotide using the DCC coupling method discussed in the previous chapter. The

analogue was then investigated for its ability to inhibit the action of the cyclase enzyme as described (section 7.6).



(**111**)

Figure 5.15 Structure of Nicotinamide Benzamide Dinucleotide (**111**).

Briefly, the effect of the addition of a known concentration of nicotinamide benzamide dinucleotide (**111**) upon the cyclisation of various different concentrations of NAD^+ was measured by monitoring the release of nicotinamide over a given time course. The experiment was repeated for four different concentrations of nicotinamide benzamide dinucleotide and the results plotted as a Lineweaver-Burk plot (figure 5.16).

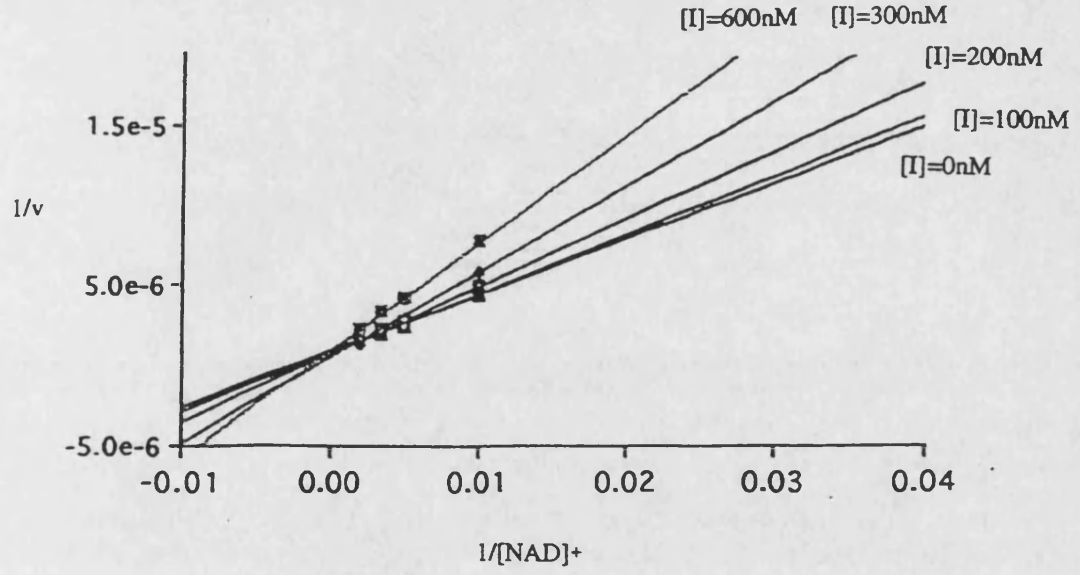


Figure 5.16 Lineweaver-Burk Plot for the Inhibition of Aplysia ADP-Ribosyl Cyclase using Nicotinamide Benzamide Dinucleotide (**111**).

The results show that nicotinamide benzamide dinucleotide is a potent competitive inhibitor of the ADP-ribosyl cyclase with a K_i value of 365nM and is the first such compound to be tested for direct inhibition of the cyclase. These results further indicate, as hypothesised, that the NMN half of the NAD^+ analogue is very important for enzyme active site recognition since this analogue, which has no adenosine like moiety is able to be competitively recognised by the active site and bind even at very low concentrations to prevent cyclisation of NAD^+.

Although this is a positive step to designing as effective inhibitor of ADP-ribosyl cyclase it has left many questions unanswered and raised many further ones. That it inhibits competitively demonstrates that NAD^+ is still able to displace nicotinamide benzamide dinucleotide from the active site at high concentrations. Additionally that during the course of these experiments no new peaks were observed by HPLC is neither indicative that benzamide diphosphate ribose (**127**) was not being formed since this compound would have a very low extinction coefficient and might not be readily observed by UV. However, what is now confirmed is that the adenine moiety is not required for enzyme recognition. This is an important lead step in the design of small molecule inhibitors for this system and further work continues to this end although it is known that NMN alone can not inhibit this active site (unpublished results). Two important target compounds to compare inhibition activities are bis-NMN (**128**) and bis-benzamide mononucleotide (**129**), structures not shown.

CHAPTER SIX

BIOLOGICAL RESULTS

6.1 Introduction

The following chapter describes the biological testing of the N1 cyclised compounds. The triethylammonium salts of the novel analogues were tested in sea urchin egg homogenate and the results were compared to those obtained with cADPR itself. Since it had already been shown that N7 cyclised compounds are inactive these analogues were not tested further.[236]

Ionic mechanisms, which regulate many cellular functions, particularly those immediately after fertilisation, have long been studied in the model system the sea urchin egg.[23] The eggs are released into the sea in an unfertilised dormant state with a depressed metabolism and they are able to survive like this for up to 48h. Upon fertilisation the eggs undergo dramatic change, triggered by two ionic events, firstly an increase of the internal pH by activation of a Na^+/H^+ exchanger in the plasma membrane and secondly an increase in the internal concentration of Ca^{2+} ions by mobilisation of ions from the internal stores. These Ca^{2+} transients can be divided into two types - the elevation caused by factors in the sperm and later transients which occur as a result of second messenger pathways operating within the cell.[240] The immediate consequence of the Ca^{2+} elevation is the formation of a proteinaceous fertilisation envelope around the egg which prevents polyspermy. In the longer term, these two changes together reactivate the cells' metabolism and set the developmental programme into motion.

Clearly the Ca^{2+} regulation systems within the sea urchin egg are complex and multiple pathways appear to be present, this makes it a good model system for the elucidation of

various different mechanisms for Ca^{2+} mobilisation. This egg also has several technical advantages. Large quantities of homogenous eggs can be obtained readily and inexpensively, at a whole cell level the eggs are large and sturdy enough to be used for micoinjection work,[25] and at a subcellular level the eggs can be prepared as an homogenate which can be stored frozen and defrosted before use with no loss of Ca^{2+} releasing activity.[24] Although this homogenate is a simple system, freed from the complex functioning of the cell as a whole, to date information obtained from this model has been applicable both to the whole sea urchin egg and to other cell systems.

6.2 Analogues of cADPR Modified in the Adenosine Ribose Ring

6.2.1 cAraDPR (113), cAcetDPR (115) and cAcycloDPR (116)

Since the adenosine ribose ring of cADPR could be involved in receptor recognition and binding it is one area of this molecule which it is important to investigate by structural modification. Initial work has already been performed within this area in this group with the synthesis and biological testing of 2'-deoxy cADPR (**34**) and 3'-deoxy cADPR (**35**).[112] Results indicate that 2'-deoxy cADPR is a good agonist for the cADPR induced Ca^{2+} release mechanism in sea urchin egg homogenates but conversely 3'-deoxy cADPR is a poor agonist (table 6.1). Additionally 3′-deoxy cADPR does not exhibit any antagonistic activity when tested in sea urchin egg homogenate.

The possibility that the poor activity of 3'-deoxy cADPR was due to poor binding was eliminated by binding studies which indicated that 3'-deoxy cADPR did competitively bind as effectively as cADPR to the binding protein. Hence, these initial studies had

indicated that the 3'-hydroxyl group was essential for good agonistic activity whilst the 2'-hydroxyl group seemed to be less important.

Compound	Compound Number	Agonist Activity (EC_{50} value)
cADPR	**7**	32±4nM
2'-deoxy cADPR	**34**	58±5nM
3'-deoxy cADPR	**35**	4.6±0.8μM

Table 6.1 Agonist Activity Data for Adenosine Ribose Modified Analogues of cADPR

In order to further study this interaction three target compounds were designed and synthesised. Firstly cAraDPR (**113**) in which the crucial 3'OH group is present but the stereochemistry about the 2'OH group had been reversed (figure 6.1), secondly cAcetDPR (**115**) in which an isopropylidene group firmly holds the two oxygen atoms of the 2′ and 3′ hydroxyl groups in a fixed conformation and also removing the possibility of these two groups donating a proton for H-bonding (figure 6.1) and cAcycloDPR (**116**) in which the ribose ring has been completely removed and replaced by a methoxy ethoxy chain (figure 6.1). In conjunction with these compounds 3'OMe-cADPR (**130**, figure 6.1), a fourth analogue in this series was designed and synthesised by a co-worker.

All of these analogues were tested in sea urchin egg homogenates for their Ca^{2+} mobilising properties and these results are shown (table 6.2).

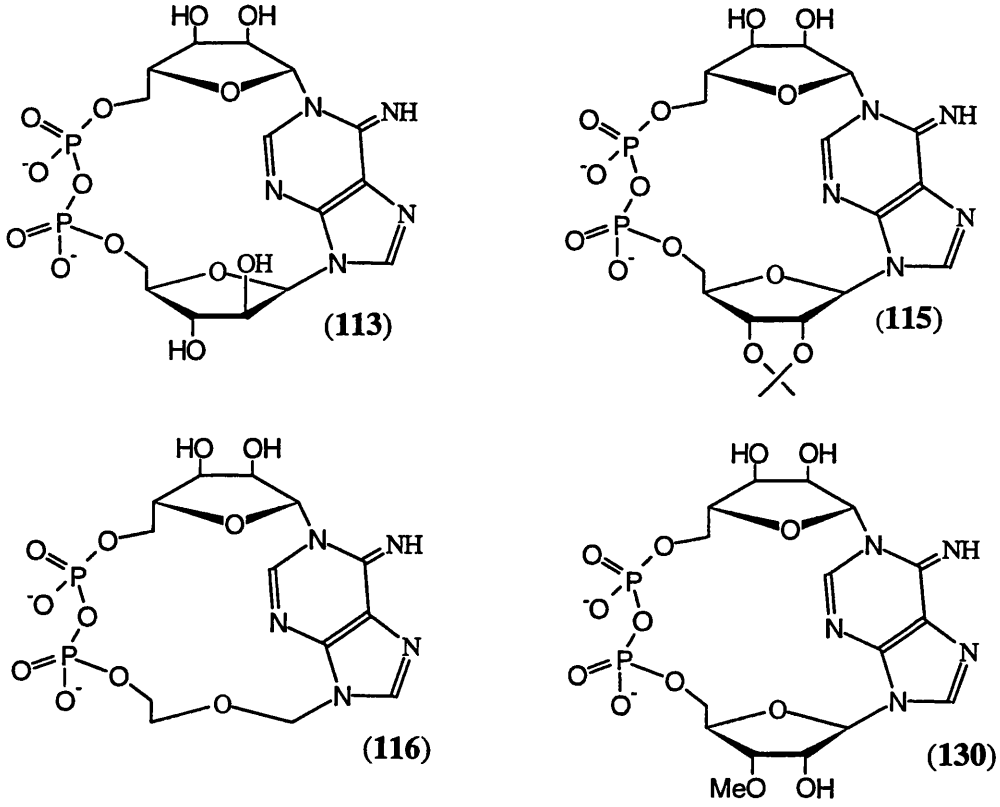
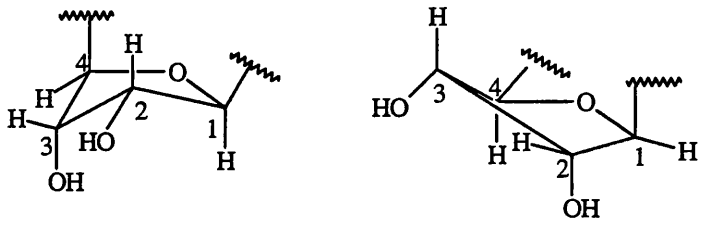


Figure 6.1 Structures of cAraDPR (**113**), cAcetDPR (**115**), cAcycloDPR (**116**) and 3'OMe-cADPR (**130**).

Compound	Compound Number	Activity (EC_{50} or IC_{50}value)
cAraDPR	**113**	inactive
cAcetDPR	**115**	poor agonist 12μM
cAcycloDPR	**116**	inactive
3'-OMe-cADPR	**130**	antagonist IC_{50}≈4.8±0.05μM

Table 6.2 Summary of the Activity Data for Adenosine Modified cADPR Analogues.

A careful study of the data for these compounds suggested that instead of there being a simple relationship between the groups attached to the ribose ring and activity the conformation of the ribose ring was of importance. In general a five-membered furanose ring is non-planar and is puckered in a twist form with three atoms in the plane and the fourth and fifth atoms displaced on opposite sides of a plane (figure 6.2).[241] The possible conformations are then named according to the atom displaced to the same side of the ring as the C5' CH_2 group namely 2'-*endo* or 3'-*endo*.



2'-endo 3'-endo

Figure 6.2 Different Conformations of Furanose Rings.

These two conformations exist in equilibrium with the 3'-endo conformation being preferred in adenosine. However, when the *purine* ring is rotated into a *syn* conformation, as it is in cADPR and 8-substiuted adenosine, then the sugar ring equilibrium changes and the ribose adopts the 2'-endo conformation. This observation highlights one of the limitations of using molecular modelling packages since our system models cADPR with this ring in a 3'-endo conformation which is in fact the opposite conformation to the correct one shown in the crystal structure (figure 6.3 where A is the crystal structure and B is the modelled structure).

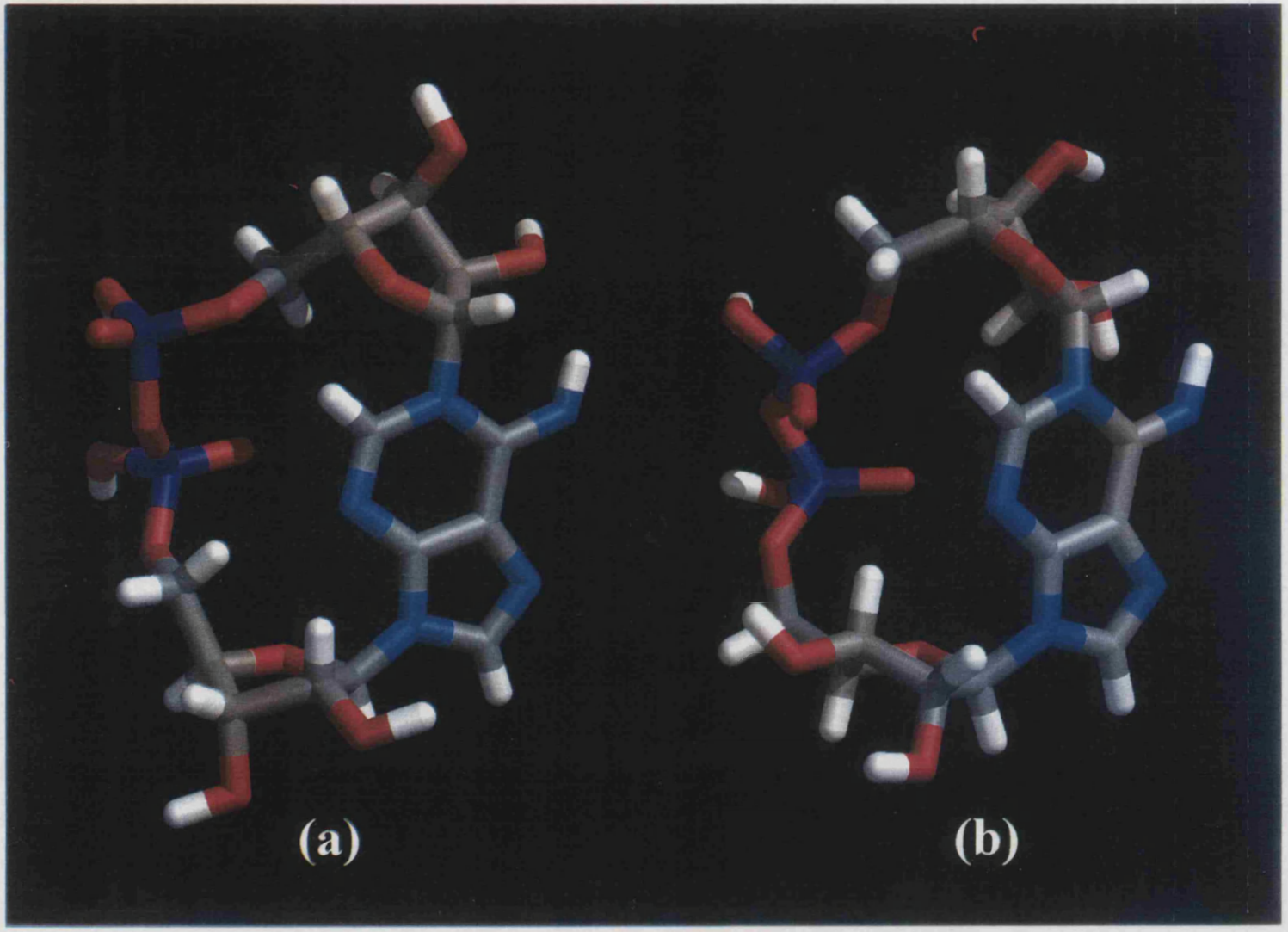


Figure 6.3 Conformations of cADPR deduced from (a) crystal structure and (b) energy-minimised molecular modelling calculations.

It is possible to determine the conformation of a ribose ring in a specific molecule by analysis of the ^1H ^1H NMR coupling constants. The size of these coupling constants is dependent upon the torsion angle between the two atoms and can be predicted using the Karplus equation.[159] In summary, as the size of this angle tends to 90° the size of the coupling constant decreases, and conversely as the angle tends to 0° or 180° the coupling constant increases. As a result very large coupling constants are observed between two ^1Hs which are orientated diaxially about a bond.

The ^1H ^1H coupling constants for the adenosine ribose rings in this series of cADPR analogues is summarised in Table 6.3. It can be seen that for all of the compounds which exhibited activity the ribose ring is in the 2'-endo conformation, as in the parent compound. However, in cAraDPR (**113**), a compound with no activity, the NMR data indicates that the ring conformation has changed to the 3'-endo structure. It thus appears that the 2'-endo ring conformation is critical to receptor activation by the analogues. Since cAraDPR (**113**) also had no antagonisitc activity it might be expected that the compound has not been able to bind to the receptor site although this has yet to be verified. The fact that cAcycloDPR (**116**) was also inactive supports the reasoning that the sugar ring conformation is critical.

Although ring conformation appears to be critical to activity testing of other analogues in this series indicates that other factors also contribute. It appears that the presence of a hydroxyl group at the 3' position is important for potent activity since upon its removal the potency of the compounds drops considerably. In cAcetDPR (**115**) the rigid nature of the 1,3-dioxolane ring slightly modifies the twist of the furanose ring shearing it to be more

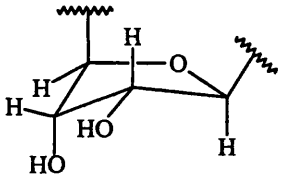
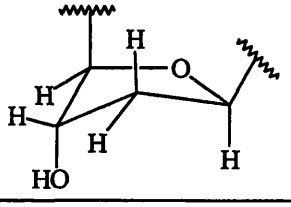
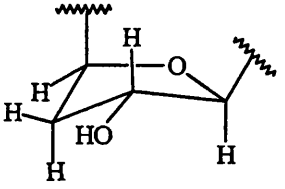
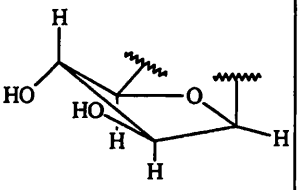
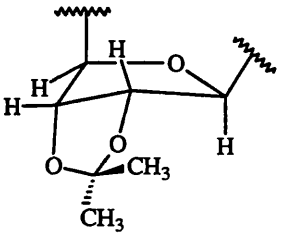
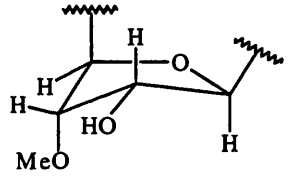
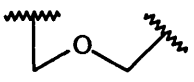
Structure	Conformation	H1'-H2' coupling	H2'-H3' coupling	Activity
cADPR **7**		5.6Hz	5.1Hz	good agonist
2'-deoxy cADPR **34**		7Hz	not determined	good agonist
3'-deoxy cADPR **35**		6Hz	not determined	poor agonist
cAraDPR **113**		7.3Hz	8.2Hz	inactive
cAcetDPR **115**		3.4Hz	6.1Hz	poor agonist
3'-OMe-cADPR **130**		5.5Hz	5.2Hz	antagonist
cAcycloDPR **116**		-	-	inactive

Table 6.3 Summary of the Coupling Constants for the Different Analogues of cADPR.

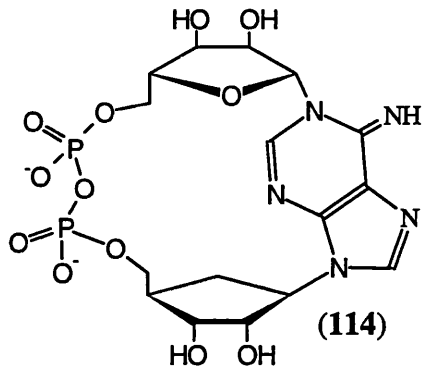
planar. This causes the oxygen atoms to lie in slightly different positions to those in the unrestrained compound and this could be the cause of the decrease of potency of the Ca^{2+} release, as could the presence of the acetal methyl groups, preventing a tight fit in the receptor site. That the analogue has any activity at all infers that the conformation is close enough to that of the parent ribose to allow for binding site recognition. However, it is possible that the decrease in activity could be caused by the fact that cAcetDPR (**113**) is no longer able to donate a proton for H-bonding. This could be further investigated with the synthesis and testing of 3'-F cADPR (**131**).

It is interesting to observe that when the 3'OH group is modified to a methoxy group this analogue (**130**) becomes a weak antagonist in sea urchin egg homogenates. This is unlikely to be a reflection upon the H-bond donating ability of the compound since cAcetDPR (**115**) is still able to induce Ca^{2+} release, but could be a reflection upon the amount of available space around this site in the sea urchin egg binding protein.

6.2.2 cArisDPR (114)

Analogues of nucleosides and nucleotides in which the furanose ring is replaced by a cyclopentyl ring have, in the past, proven to be good mimics of the parent compounds often with additional eg antiviral properties.[242] Hence, as part of this project, the first carbocylic analogue of cADPR was synthesised wherein the adenosine ribosyl moiety was replaced with a carbocyclic 5-membered ring to give cArisDPR (figure 6.4).

cArisDPR was tested for its concentration dependent Ca^{2+} mobilising ability in a comparative study with cADPR.[243] The carbocyclic analogue was shown to release Ca^{2+} with a potency which was only slightly lower than for the parent compound, EC_{50} values are 30nM and 80nM for cADPR (**7**) and cArisDPR (**114**) respectively.



(**114**)

Figure 6.4 Structure of cArisDPR (**114**).

Interestingly though, when the hydrolysis of this new analogue was compared with that of cADPR exciting results were obtained. A time course for the degradation of the two samples was measured by pre-incubation of the analogue with homogenate and then removal of an aliquot and testing this for its Ca^{2+} releasing ability with further homogenate. In this system cADPR was seen to rapidly degrade with its Ca^{2+} releasing ability falling to near negligible values over approximately 25min. There was also a significant decrease in releasing potency of over 70% in the first 10min. In contrast, the time course degradation of cArisDPR (**114**) was markedly slower with only a 10% reduction in releasing ability in the first 10min and a significant Ca^{2+} release still observable after 5h (figure 6.5).

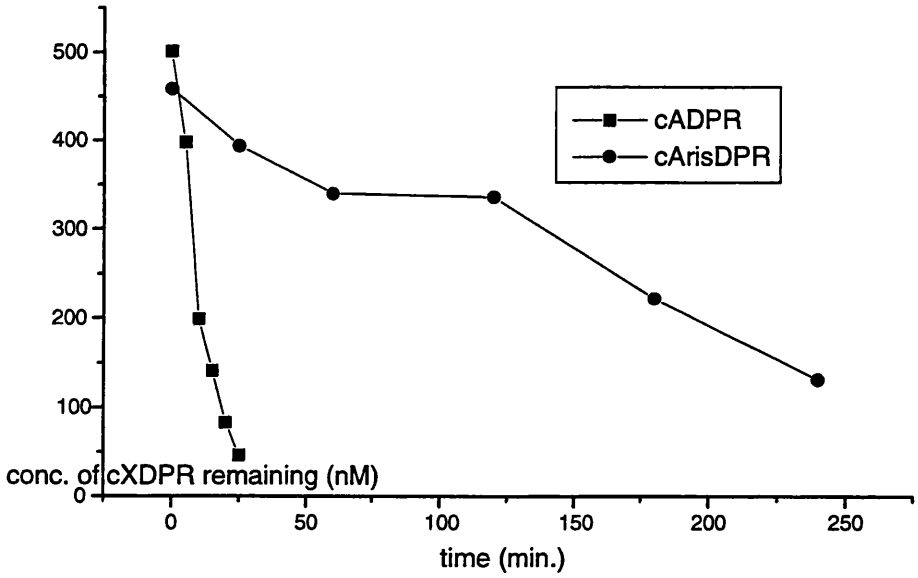


Figure 6.5 Degradation of cADPR (**7**) and cArisDPR (**114**) when Subjected to Enzyme Mediated Hydrolysis.

This increase in hydrolytic stability towards the degrading enzyme, conferred by the replacement of the adenosine ribose with a carbocyclic ring, is surprising since it is the N1-ribosyl linkage which is cleaved in the catabolism of cADPR. At the time it was proposed that this was due to the analogue binding to the degradation enzyme active site in a similar, but subtly different, conformation to that of cADPR, making the hydrolytic attack of water more difficult although clearly not impossible. With the advent of the knowledge discussed above that the ring conformation of this ribose ring is critically important to activity, it could also be the case that it is important for binding to the hydrolase enzyme active site. The cyclopentyl ring obviously adopts a conformation which reasonably mimics the parent compound, since it is able to induce Ca^{2+} release, but its slightly lower potency might be a reflection upon a slight change in shape. It is this shape which could also be critical for efficient hydrolase activity. Unfortunately, it is not

possible to confirm the conformation of cyclopentyl rings in the same manner as used for the furanose since the ^1H ^1H coupling constants are complicated by the protons at the C6' position.

The development of a poorly hydrolysable analogue of cADPR (**7**) such as cArisDPR (**114**), and ultimately completely non-hydrolysable compounds, allows the potentially wider role of cADPR induced Ca^{2+} signalling to be more easily explored in cells/tissues which express high hydrolase activity eg neuronal tissue, and where it has not, as yet, been possible to confirm the presence of cADPR-mediated Ca^{2+}-releasing pathway. Preliminary experiments have been performed in cardiac muscle where CICR, thought to be mediated by cADPR,[72] prolongs the action potential and hence the force of contraction of the cell. It has recently been demonstrated that 8-amino cADPR (**11**) is able to antagonise this CICR providing good evidence that this release is indeed mediated by cADPR, but as yet it has not been possible to increase this Ca^{2+} transient by addition of cADPR. However, when cArisDPR (**114**) was added to intact cardiac myocytes the peak magnitude of the cell contraction was shown to increase when compared to those following addition of cADPR (figure 6.6). The poor result obtained following the addition of cADPR is thought to result from the rapid degradation of cADPR following injection into the myocytes. This is the first example of cArisDPR (**114**) being used to obtain an agonist response from the cADPR Ca^{2+}-release system in cells where cADPR is degraded too rapidly to clearly demonstrate activity.

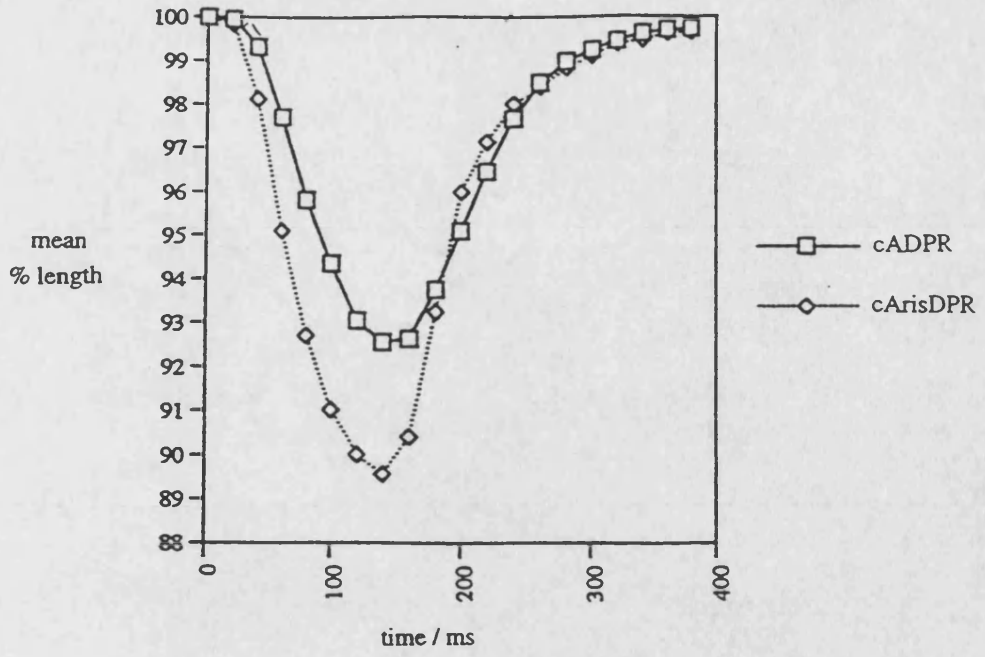


Figure 6.6 Mean Contraction of Cardiac Muscle is Extended Further by the Addition of cArisDPR (**114**) than by cADPR (**7**).

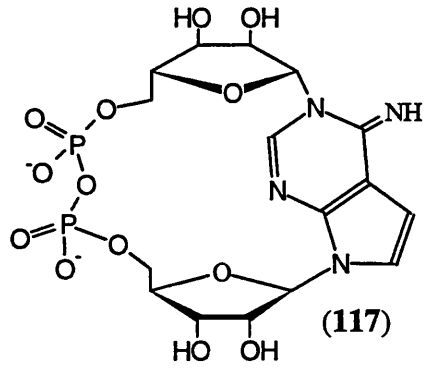
Despite being resistant to enzyme mediated hydrolysis this analogue would not be expected to be any more resistant to chemical induced hydrolysis than cADPR since the modification is in a position remote from the site of hydrolytic cleavage. This is a severe weakness in the design since it is chemical hydrolysis which prevents those pharmacological manipulations which require the compounds to survive in solution at elevated temperatures. One way in which this problem could be overcome would be to replace the northern ribose ring with a cyclopentyl hydroxyl substituted ring thus stabilising the hydrolytically labile N1-ribose linkage. This is a challenging chemical problem which involves modifying the NMN half of the NAD^+ analogue. However recent work by Hutchinson *et al.* [246] have managed to synthesise such a β-NAD^+ analogue although no results have been reported concerning its enzyme cyclisation or biological evaluation.

6.3 Analogues of cADPR Modified in the Purine Ring

6.3.1 7-Deaza-cADPR (117)

Te first analogues of cADPR to be synthesised were compounds which were substituted at the 8-position.[67] When tested these compounds were shown to be competitive antagonists to the cADPR Ca^{2+} release system and it was therefore immediately obvious that this position was crucial for activity. To further understand this activity requirement it is of interest to study other chemical modifications at the 8-position and at adjacent positions.

With this aim in mind a target compound was designed in which the nitrogen at the 7-position of the imidazole ring was replaced with a CH group to give 7-deaza cADPR (figure 6.7). This modification would allow the effects of altering the purine ring to be investigated and also begin to analyse the requirement of any hydrogen bonding in this region.



(117)

Figure 6.7 Structure of 7-Deaza-cADPR (**117**).

The parent nucleoside, 7-deazaadenosine is commonly called Tubercidin. It was isolated in 1957 by Anzai and co-workers from the fermentation broth of *Streptomyces tubercidicus*.[245] Within cells Tubercidin is known to undergo some of the usual

biochemical transformations, for example it is rapidly phosphorylated to the triphosphate derivatives [246] but it also exhibits antibiotic properties. It has been shown to inhibit the growth of several organisms [247] and to inhibit DNA, RNA and protein synthesis in several cell lines including human tumours.[248] Given that 7-deazaadenosine (**58**) interacts with biological systems it appeared that 7-deaza NAD^+ (**107**) was a target compound which was likely to be accepted by ADP-ribosyl cyclase for cyclisation and that the cyclic product would have some biological activity.

7-Deaza-cADPR (**117**) was tested in a comparative study with cADPR (**7**) for its pharmacological characteristics in sea urchin egg homogenate as described in the experimental (section 7.7.2).[249] It was capable of releasing Ca^{2+} from internal stores in a concentration dependent manner but the amount of Ca^{2+} released was consistently lower than that released by the same concentration of the parent compound (figure 6.8) with EC_{50} values of cADPR (**7**) and 7-deaza-cADPR (**117**) being 25$\pm$2nM; S.E.M. n=3, and 90$\pm$4nM; S.E.M. n=3 respectively. At maximal concentrations 7-deaza-cADPR (**117**) was only able to release around 66% of Ca^{2+} mobilised by a maximal concentration of cADPR. That this effect was not caused by enhanced hydrolysis of the compound by cADPR hydrolase enzymes was investigated, by incubating the sample with homogenate for the times indicated and then testing for its Ca^{2+} releasing ability. In fact the reverse was shown to be true, namely that the half-time for degradation of 7-deaza-cADPR (**117**) was considerably longer than for cADPR (**7**) which indicated resistance to hydrolysis by cADPR hydrolases (figure 6.9).

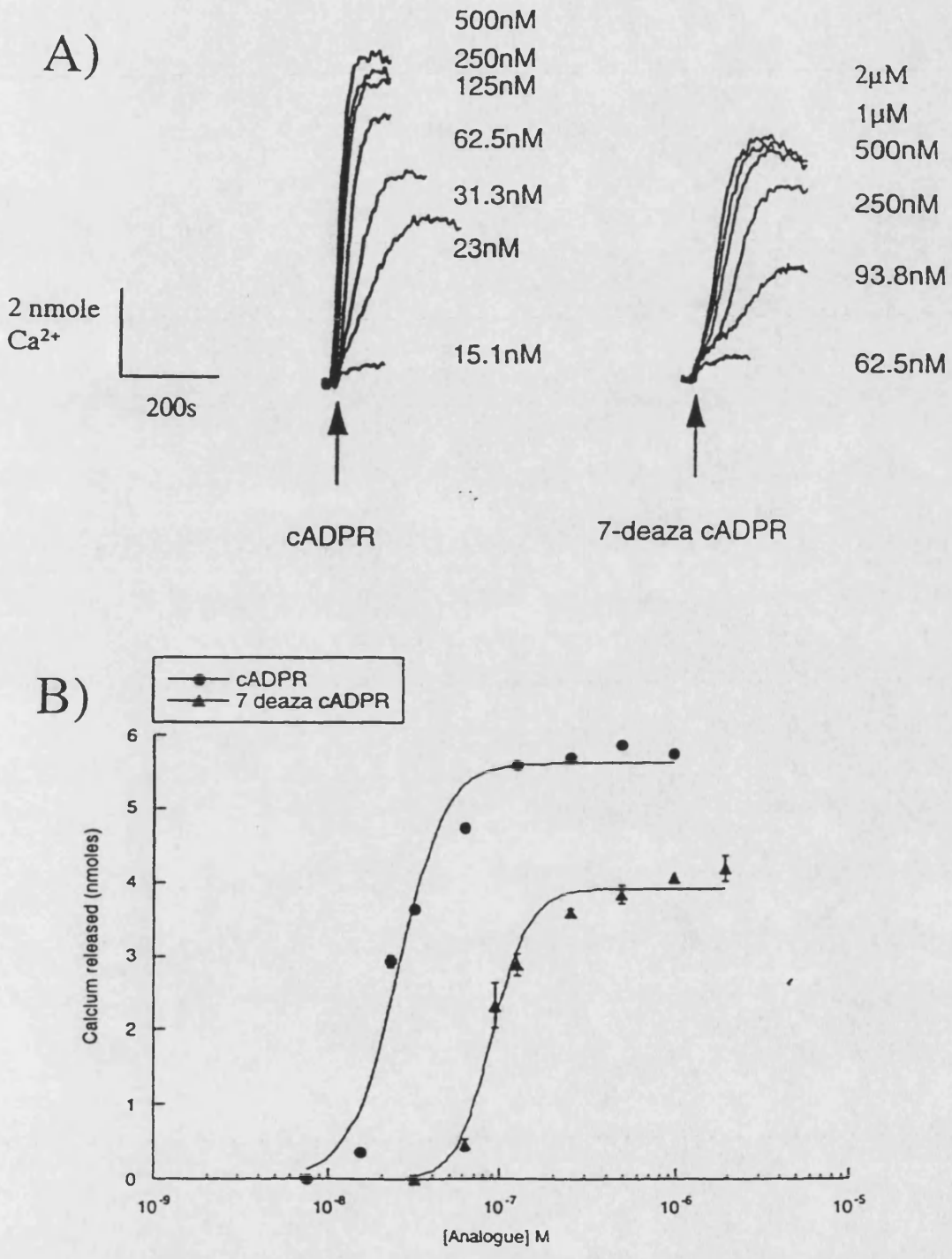


Figure 6.8 Ca^{2+}-Releasing Action of cADPR (**7**) and 7-Deaza-cADPR (**117**).

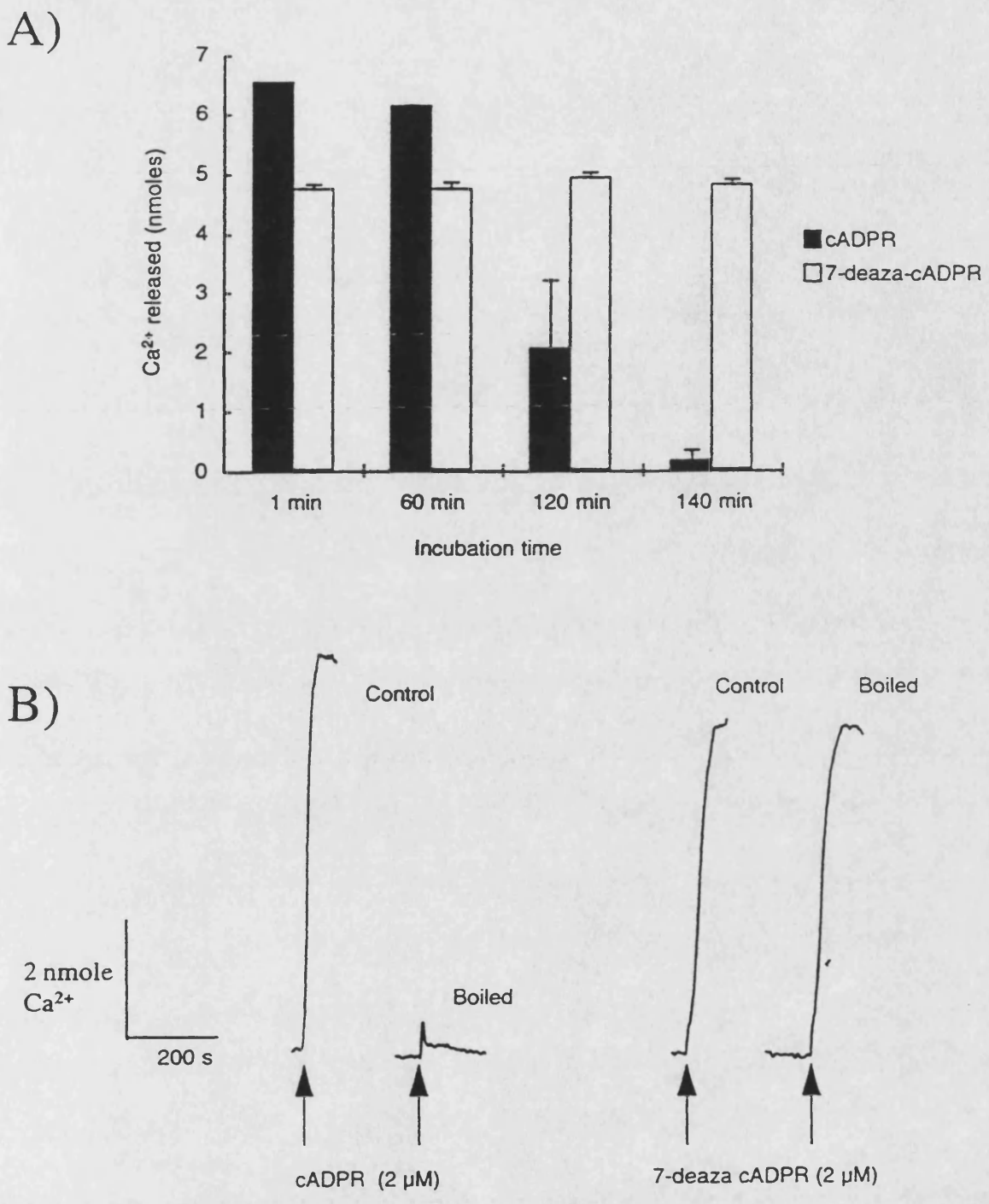
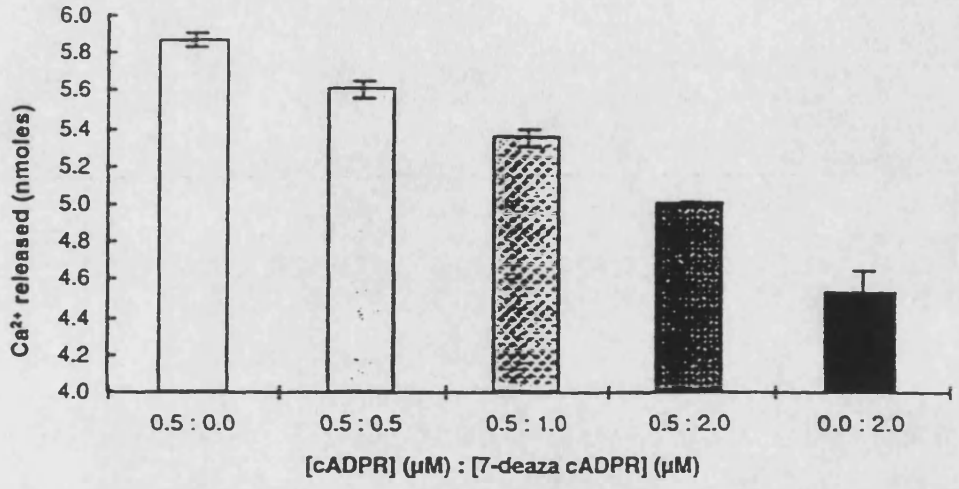


Figure 6.9 Stability of 7-Deaza-cADPR (**117**). (A) cADPR and 7-Deaza-cADPR were Incubated with Egg Homogenate. At the times Shown a Sample was Tested for its Ability to Release Ca^{2+} from a Second Aliquot of Homogenate. (B) Action of Heat Induced Hydrolysis on the Ca^{2+}-Mobilising Activity of cADPR and 7-Deaza-cADPR.

The lower efficacy of 7-deaza-cADPR (**117**) indicated that the analogue might be a partial agonist of the cADPR (**7**) release system, an activity which had not yet been reported and this was tested by examining the ability of 7-deaza-cADPR (**117**) to inhibit the maximal Ca^{2+} mobilising properties of cADPR (**7**) itself. Figure 6.10A shows that the maximal Ca^{2+}-released by cADPR (**7**) alone is decreased by increasing the concentration of 7-deaza-cADPR (**117**). Hence 7-deaza-cADPR (**117**) and cADPR (**7**) can progressively compete for Ca^{2+}-release activity and cADPR (**7**) can eventually overcome the inhibition produced by a maximally effective concentration of 7-deaza-cADPR (**117**). However, combinations of submaximal concentrations of cADPR (**7**) and 7-deaza-cADPR (**117**) appeared to be additive (figure 6.10B) which mitigates against the possible formation of an inhibitory metabolite on cADPR-induced Ca^{2+}-release. These data are consistent with a partial agonist action of 7-deaza-cADPR (**117**).

It was shown that 7-deaza-cADPR (**117**) and cADPR (**7**) were acting at the same Ca^{2+}-release channel by cross desensitisation experiments. Homogenate pre-treated with a supramaximal dose of cADPR (**7**, 1μM) showed no further Ca^{2+} release when subsequently treated with 7-deaza-cADPR (**117**, 500nM) and vice versa (figure 6.11). In addition, when homogenate was treated with 8-amino cADPR (**11**, 250nM), a competitive antagonist of this system, no further Ca^{2+}-release was elicited by a subsequent addition of either cADPR (**7**, 500nM) or 7-deaza-cADPR (**117**, 500nM). That 7-deaza-cADPR (**117**) and cADPR (**7**) interact with the same binding site was further confirmed by binding studies of [^{3}H]-cADPR (**132**) to sea urchin egg homogenates. 7-Deaza-cADPR (**117**), like cADPR (**7**), was equally effective at reducing radioligand binding at comparable concentrations indicating similar affinities for the same binding site (figure 6.12).

A)



B)

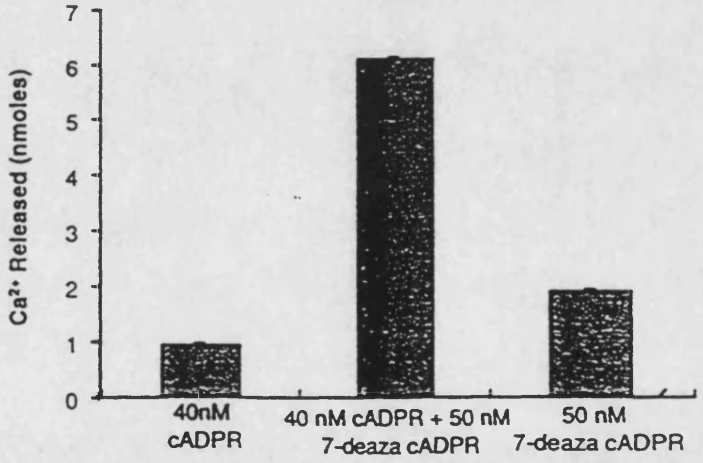


Figure 6.10 Action of 7-Deaze-cADPR (**117**) as Partial Agonist. (A) The Amount of Ca^{2+}-Released by cADPR can be Reduced by Adding Increasing Concentraions of 7-Deaza-cADPR. (B) The Effect of Co-Addition of Submaximal Doses of the Agonists.

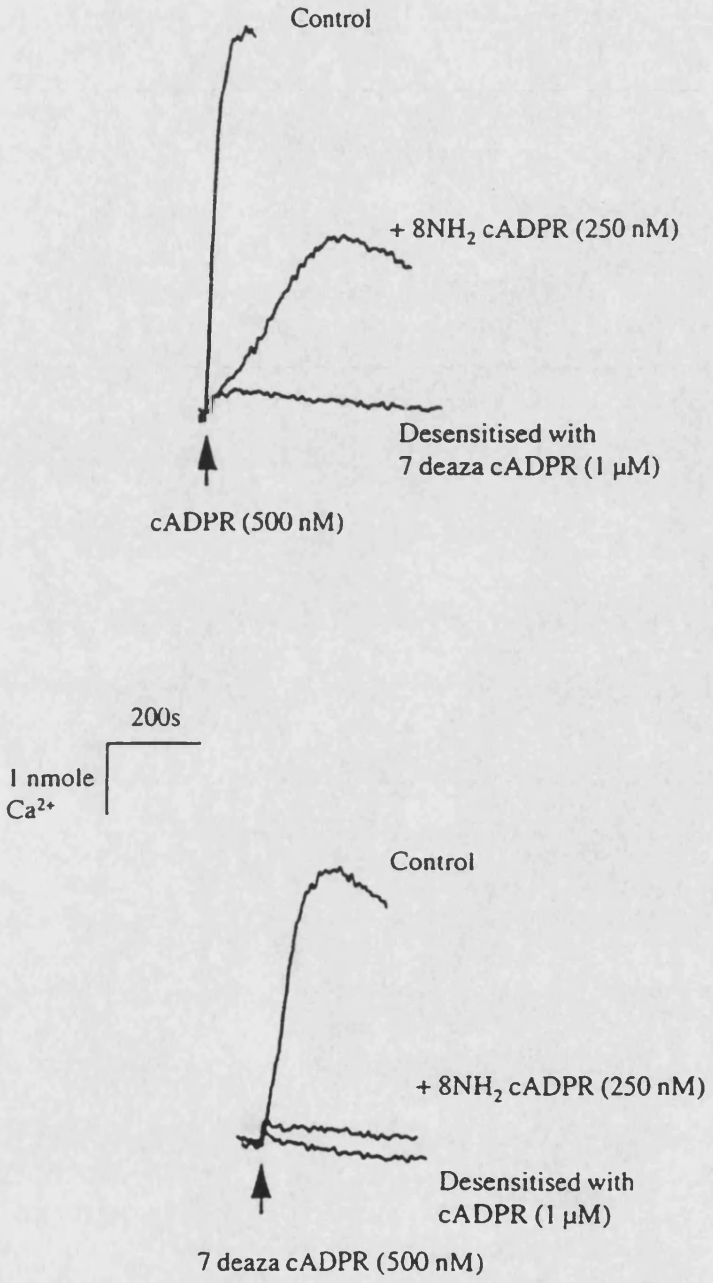


Figure 6.11 That 7-Deaza-cADPR (**117**) Acts on cADPR Sensitive Channels is Demonstrated by Cross Desensitisation Experiments.

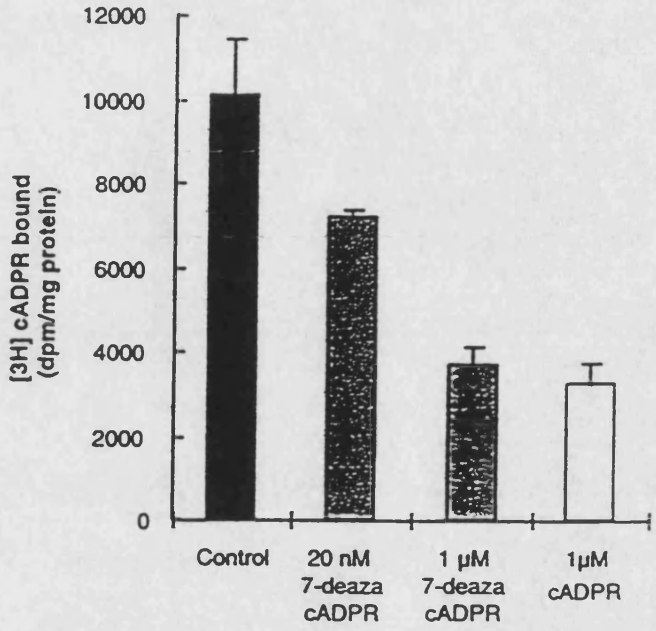


Figure 6.12 7-Deaza-cADPR (**117**) is Equally as Effective at Displacing [^{3}H]-cADPR as cADPR (**7**) from Binding Sites in Sea Urchin Eggs.

That 7-deaza-cADPR (**117**) is able to bind and release Ca^{2+} indicates that the N-7 is not essential for receptor binding and activation, however, its absence clearly interferes with the Ca^{2+} release machinery such that a sub-maximal level of Ca^{2+} is released. Although this can not be conclusively rationalised at a molecular level this change in activity may be a reflection of the requirement for hydrogen bonding between the N-7 position of the adenine and the binding protein. The 7-deaza compound might be expected to present itself to the receptor in a similar way to cADPR, albeit now lacking one hydrogen bond

interaction and potentially interfering sterically with a putative hydrogen bond donor, residue X (figure 6.13).

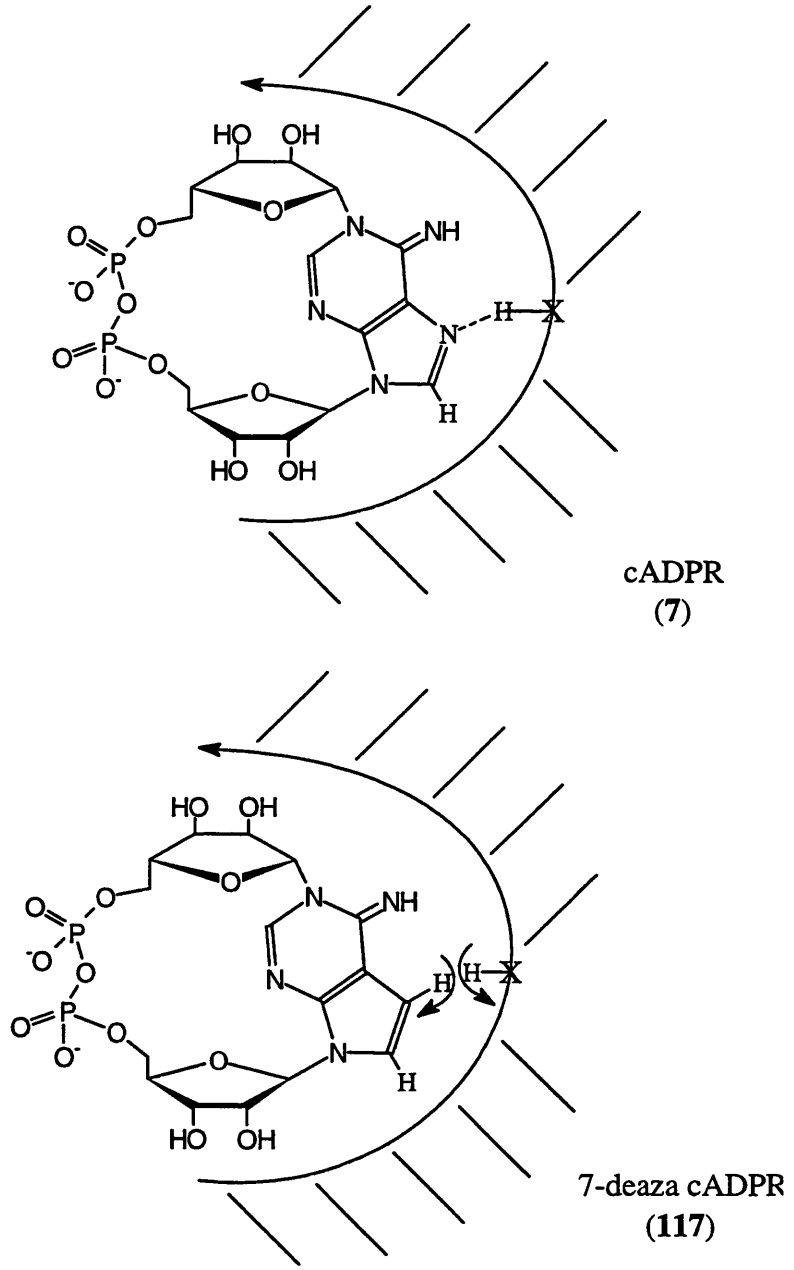


Figure 6.13 Hypothetical cADPR-Binding Protein Interaction Illustrating a Possible Loss of a Hydrogen Bond when 7-Deaza-cADPR (**117**) occupies the Binding Site.

However, in the case of the antagonist 8-amino cADPR (**11**) perhaps the amino group causes binding to the receptor in a different way, possibly with the amino group competing with the N-7 for this putative H-bonding interaction, thus shearing the molecule into an unfavourable conformation for the stimulation of ion-channel opening. Clearly, these considerations must operate within the limitations of steric constraint at the 8 position since it is known that as the 8-substituent increases in steric bulk there is a reduction in the antagonistic activity.[250]

The results which indicate that this analogue is resistant to chemical hydrolysis also have important pharmacological implications. In the literature there seems to be some dispute as to whether cADPR and its analogues are suitable for microinjection work. Perez-Terzic and co-workers [251] clearly state that 8-amino cADPR (**11**) is unsuitable for microinjection due to its chemical instability. However, Lee *et al.* have used microinjection techniques to introduce 8-amino cADPR (**11**) into sea urchin eggs and have observed the blocking of cADPR-induced Ca^{2+} release.[110] It is interesting to note that Webb *et al.* [252] used microinjection techniques to look at the action of 8-amino cADPR (**11**) on the increase in intracellular Ca^{2+} in response to glucose. Using this technique they were unable to observe any antagonism of the response and concluded that cADPR induced Ca^{2+} release did not play a role in glucose stimulated Ca^{2+} release. This is in stark contrast to other work where cADPR (**7**) has clearly been shown to play a role (section 1.7.2) and this begs these question as to whether these anomalous results occurred due to the stability of the antagonist and the techniques used. However, a compound which was resistant to chemical hydrolysis, could definitely be used for microinjection and would

therefore allow further intact cell investigations to be made on the cADPR-induced Ca^{2+} mechanism.

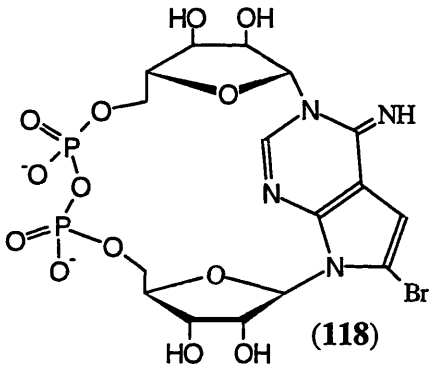
It has previously been reported that in 7-deaza analogues of adenosine the glycosidic bond is much more resistant to acid catalysed hydrolysis than in the corresponding adenosine analogues.[253] Similarly the increased hydrolytic resistance of 7-deaza analogues of cADPR was thought to arise from an increased stability of the N1 ribosyl linkage. It is known that the N1 position of 7-deazaadenosine is more basic than that of adenosine (pKa values are 5.2 and 3.6 respectively [254]) and in an argument analogous to that used previously (section 5.2.1) that N1 in a 7-deaza purine ring is more basic than adenine this will be a worse leaving group and therefore it will be harder to cleave the N1-ribosyl ring.

Although more work is needed to fully understand the structural requirement for antagonism these results show that it is not just the 8-position of the imidazole ring which is important for activity. The next step was to introduce a combined modification between the 7- and 8-positions to observe the effects of this on antagonism and to try to confer hydrolytic stability to the antagonists.

6.3.2 7-Deaza-8-Bromo-cADPR (118)

As discussed in the previous section, the combination of the replacement of the 7-position nitrogen with a CH group and a substitution at the 8-position should lead to a non-hydrolysable antagonist with potentially better antagonist activity than its 7-aza counterpart. Although it is known that 8-amino cADPR (**11**) is a better antagonist than 8-

bromo cADPR (**27**) it was decided that the 8-bromo analogue, with its shorter chemical synthesis, would be a better target with which to check the biological activity hypothesis. It was with these aims in mind that 7-deaza-8-bromo-cADPR (**118**) was synthesised (figure 6.14).[255]



(**118**)

Figure 6.14 Structure of 7-Deaza-8-Bromo-cADPR (**118**).

When tested in sea urchin egg homogenate 7-deaza-8-bromo-cADPR (**118**), similarly to 8-bromo cADPR (**27**), did not induce any Ca^{2+} release even at concentrations which were well in excess of concentrations of cADPR (**7**) or 7-deaza-cADPR (**117**) needed to elicit maximal release (figure 6.15).[256]

7-Deaza-8-bromo-cADPR (**118**) was then tested for its antagonistic activity. Sea urchin egg homogenates pretreated with this analogue were no longer responsive to 100nM cADPR (**7**), results consistent with the activity of 8-bromo cADPR (**27**). For both compounds this competitive antagonism was dependent upon the antagonist concentration with IC_{50} values of 0.73±0.05μM and 0.97±0.04μM for 7-deaza-8-bromo-cADPR and 8-bromo cADPR respectively. The removal of the 7-position N atom had therefore made this new antagonist marginally better in its activity (figure 6.16).

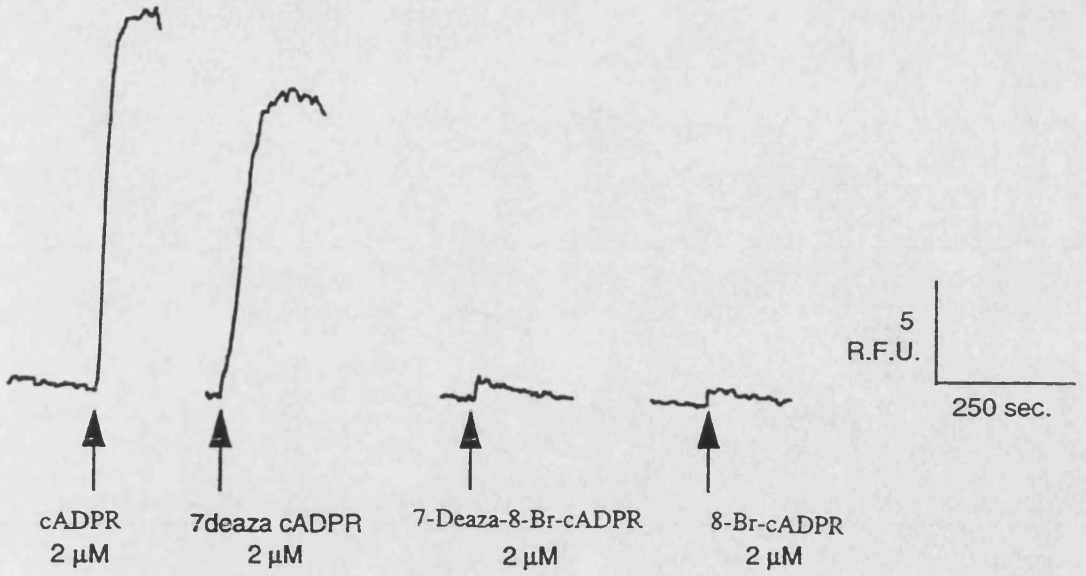


Figure 6.15 Ca^{2+} Releasing Action of cADPR (**7**), 7-Deaza-cADPR (**117**), 8-Br-cADPR (**27**) and 7-Deaza-8-Br-cADPR (**118**).

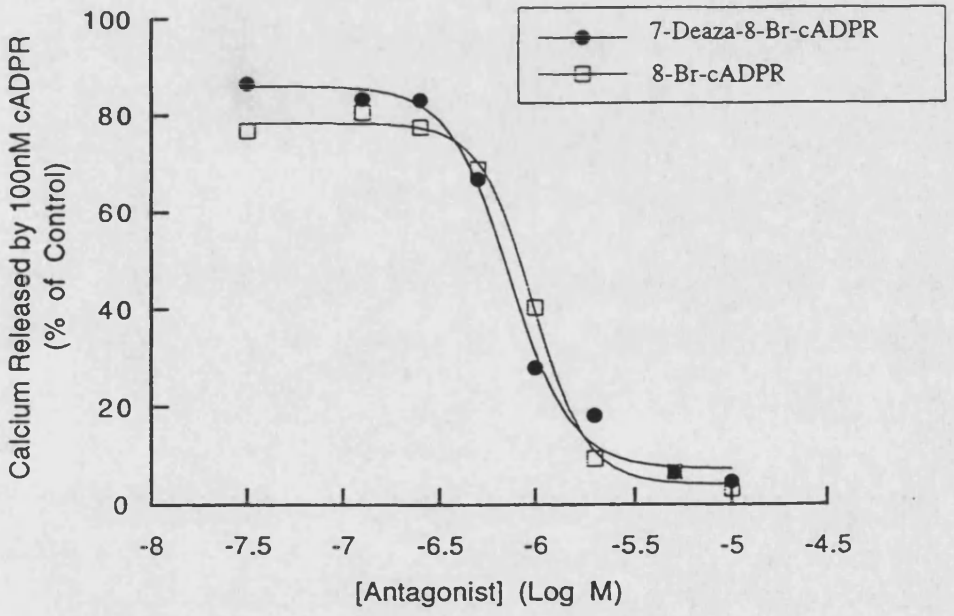


Figure 6.16 Concentration Dependent Inhibition of cADPR-Induced Ca^{2+} Release by 8-Br-cADPR (**27**) and 7-Deaza-8-Br-cADPR (**118**).

To test whether the removal of the N7 atom had conferred hydrolytic stability to this antagonist standard solutions of both the 8-bromo cADPR (**27**) and 7-deaza-8-bromo-cADPR (**118**) were subjected to heat induced hydrolysis, a treatment which has previously been shown to effectively hydrolyse unstable compounds.[72] After heating to 80°C for 90min an aliquot of the 8-bromo cADPR (**27**) solution was no longer able to antagonise the Ca^{2+} mobilising properties of cADPR. Importantly, an aliquot of the 7-deaza-8-bromo-cADPR solution (**118**), which had been treated in the same manner, was still able to antagonise cADPR (**7**) release (figure 6.17) and HPLC analysis of these samples confirmed the degradation of 8-bromo cADPR (**27**) whilst 7-deaza-8-bromocADPR (**118**) was still present.

When tested for resistance to enzyme mediated hydrolysis similar results were obtained. Egg homogenates were incubated overnight at 17°C with 20μM solutions of either antagonist. Samples were then taken and tested for antagonistic activity on cADPR-induced Ca^{2+} release. Whereas the antagonistic activity of 8-bromo cADPR (**27**) had dramatically reduced following the overnight incubation (65.8±8.3% of control cADPR (**7**) release was achieved n=3), the level of 7-deaza-8-bromo-cADPR (**118**) induced antagonism remained high (28.5±11.4% of control cADPR release was achieved n=3), implying that enzyme induced hydrolysis of this sample had been somewhat slower. Unfortunately the presence of the cyclic analogues in these samples could not be confirmed by HPLC due to the presence of other UV active material which obscured the required peaks. These data indicate that 7-deaza-8-bromo-cADPR (**118**) is the first hydrolytically stable antagonist of cADPR (**7**). The argument used to explain the hydrolytic stability of 7-deaza cADPR is still appropriate when discussing this analogue.

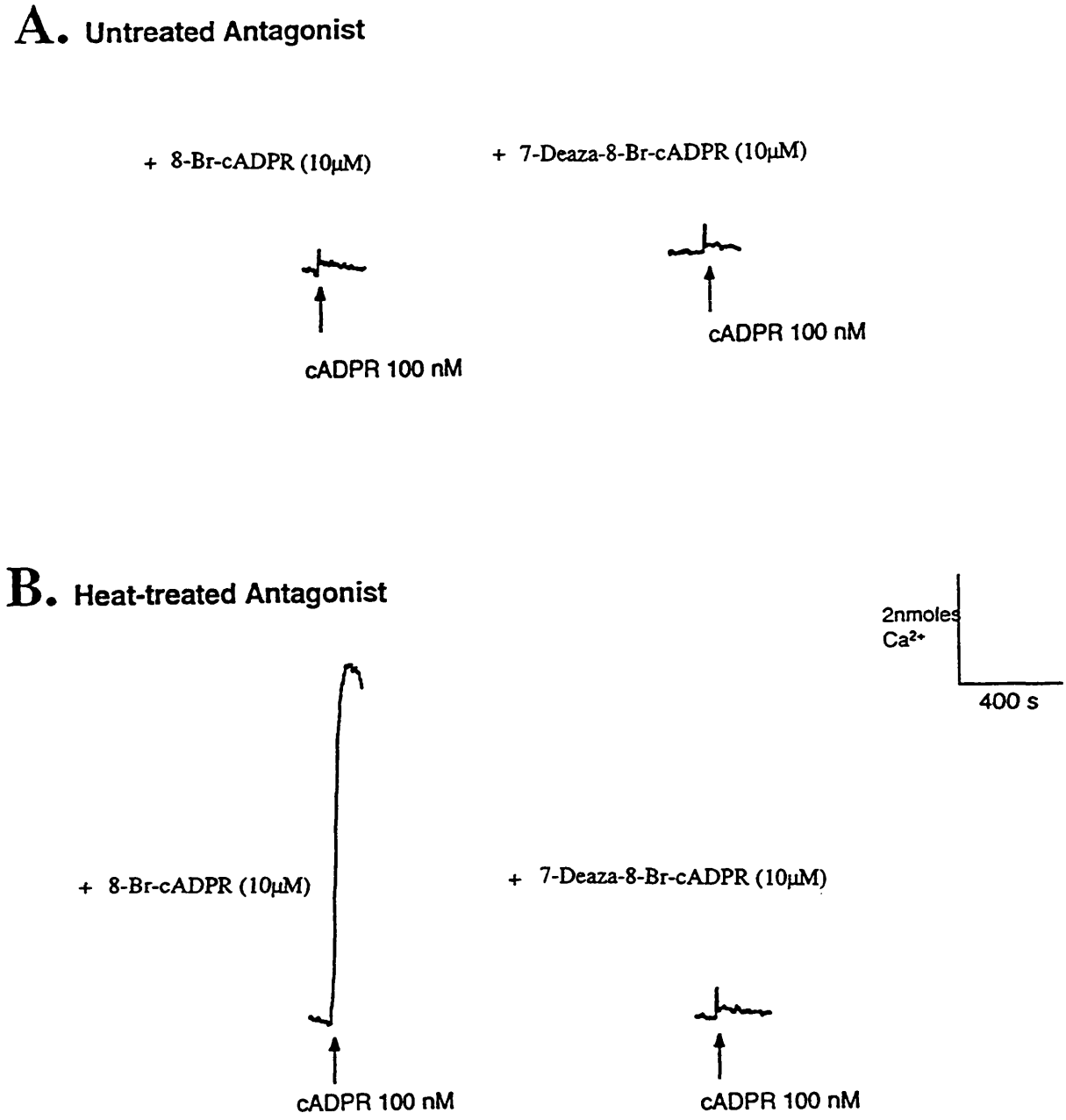


Figure 6.17 Effect of Heat Treatment on Antagonistic Activity of 8-Br-cADPR (**27**) and 7-Deaza-8-Br-cADPR (**118**).

The N1 position of 8-bromo adenosine is more basic than in the unsubstituted material (pKa value is 4.02 for 8-bromo adenosine [257]) and thus combination of both substitution at the 8-position and removal of the N7 nitrogen makes this purine ring substantially more basic than adenine. Hence this ring is an even worse leaving group meaning that the cyclic analogue is even less likely to be destroyed by hydrolysis than cADPR.

Tubercidin and Tubercidin 5'-monophosphate are known to be readily absorbed into red blood cells [258] and it has also been shown that 8-bromo cGMP (**133**) is membrane permeable.[259] It was therefore possible that these two lipophilic modifications to 7-deaza-8-bromo-cADPR (**118**) could have made this analogue membrane permeable. This hypothesis was investigated by monitoring the effect of extracellular applications of 7-deaza-8-bromo-cADPR (**118**) on the fertilisation induced Ca^{2+} waves in intact sea urchin eggs. Eggs, which had been injected with the $Ins(1,4,5)P_3$ antagonist heparin (250μg/ml) and a Ca^{2+} sensitive dye, fura-2 (2μM), exhibited a propagating Ca^{2+} wave upon addition of sperm. This wave had an average amplitude of 1705±119μM Ca^{2+} (n=11) and took 41.9±5.8s (n=11) to reach this peak, results which are consistent with the previously reported observations of sperm induced Ca^{2+} mobilisation.[60] However, when heparinised eggs were pre-incubated in a bathing solution containing 7-deaza-8-bromo-cADPR (50μM) the sperm induced transients were significantly reduced, the wave amplitude now being 988±81μM Ca^{2+} (n=5), and took significantly longer to reach this peak (69.4±6.8s, n=5). This inhibition of fertilisation induced Ca^{2+} mobilisation was also dependent on antagonist concentration (figure 6.18).

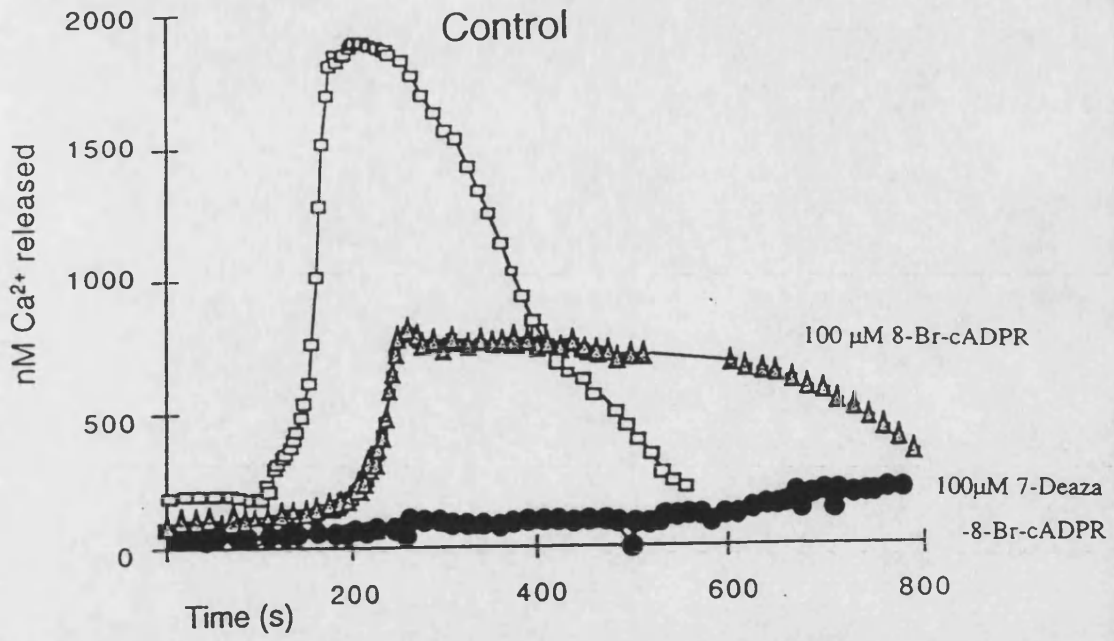


Figure 6.18 (A) Antagonistic Actions of Extracellularly Applied 8-Br-cADPR (**27**) and 7-Deaza-8-Bromo-cADPR (**118**) on Fertilisation Induced Ca^{2+} Transients in Intact Sea Urchin Eggs - Control Response and Reduction Upon Addition of Antagonist.

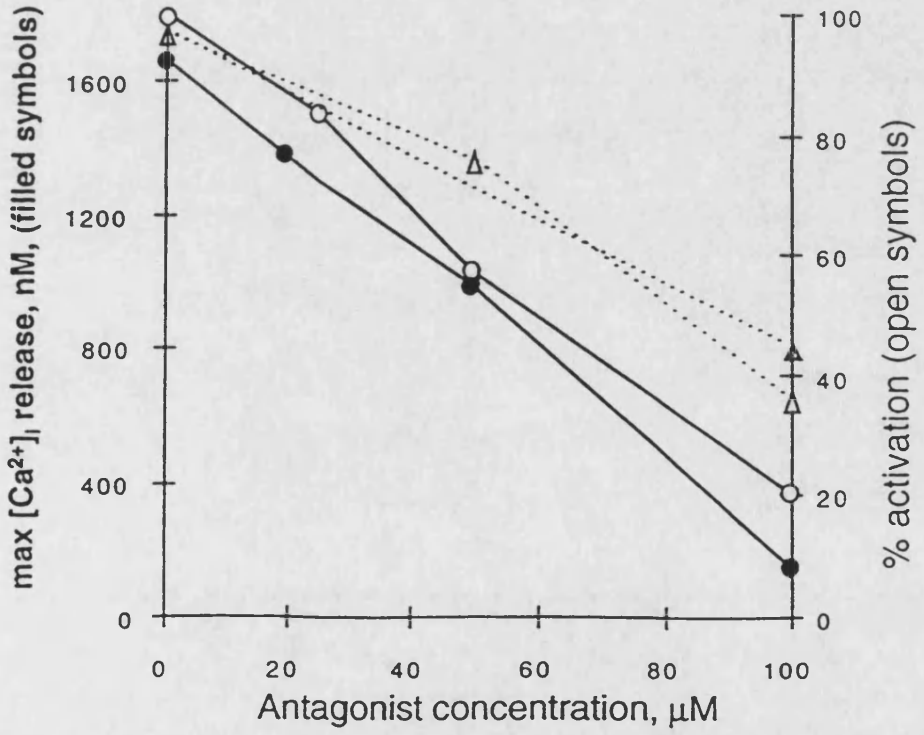


Figure 6.18 (B) Dose Depedent Action of 7-Deaza-8-Br-cADPR (**118**,) and 8-Br-cADPR (**27**, Δ)on Peak Fertilisation Induced Ca^{2+} Rise and Egg Activation. Both of these Antagonists can Reduce the number of Activated Eggs but 7-Deaza-8-Br-cADPR is more Effective at all Concentrations.

Intracellular Ca^{2+} mobilisation in sea urchin eggs following fertilisation is a pre-requisite for the formation of a cortical envelop to prevent polyspermy [37] and the prevention of the formation of such an envelope is an indication of the antagonism of the sperm induced Ca^{2+} transient. Treatment of heparinized eggs with 7-deaza-8-bromo-cADPR (**118**) did indeed prevent this cortical reaction following fertilisation, whereas the reaction was not prevented in similarly treated eggs in which the Ins(1,4,5)P_3 Ca^{2+} release had not been antagonised with heparin. This suggests that the presence of 7-deaza-8-bromo-cADPR (**118**) does not effect sperm activity but that the analogue is able to permeate the sea urchin egg plasma membrane and specifically compete for endogenous cADPR-binding sites thus inhibiting cADPR induced Ca^{2+} release.

This analogue is the first membrane permeable, non-hydrolysable antagonist of cADPR induced Ca^{2+} release and is a very important pharmacological tool. Since its lipophilic properties confer membrane permeability the need for potentially disruptive protocols such as cell permeabilisaton and micro-injection methods to introduce the antagonist into the intact cell is eliminated. This analogue will therefore allow investigations of the role of cADPR in physiological response to extracellular stimuli to be carried out more effectively.

6.4 Conclusions

This thesis set out with the objective to develop, and utilise, a simple chemo-enzymatic route for the production of analogues of cADPR and then to test them for their activity in order to further develop a structure activity profile for the mechanism of action of cADPR.

The chemistry route that has been developed and utilised is such that any analogue of adenosine can be synthesised, regioselectively phosphorylated without the need for extensive selective protection and coupled with NMN to give an analogue of β-NAD^+. This compound is then cyclised to give a modified cADPR product using *Aplysia* cyclase. By challenging the enzyme with substrates altered in both the ribose rings and the purine ring this thesis has further confirmed, in general, the loose substrate specificity of this enzyme with the synthesis of 6 novel analogues of cADPR.

The novel analogues of cADPR were tested for their activity in the cADPR induced Ca^{2+} release mechanism. Those modified in the adenosine ribose added to the information already known about this position and have led to the important hypothesis that the conformation of this ring is critical for interaction with the binding protein and thus activity. These analogues have also confirmed that the 3'OH is also essential for a good agonist response. Additionally in this series the first analogue of cADPR with increased stability against enzyme mediated hydrolysis was synthesised by replacing the adenosine ribose with an appropriately substituted cyclopentyl ring. The only explanation to date to explain this result, since the modification is so far removed from the site of hydrolysis, is

to again focus attention upon the conformation of this five membered ring with the implication that it is also important for binding to the active site of the hydrolase.

The first partial agonist of this Ca^{2+} release mechanism was synthesised as a result of this work by replacement of the N7 atom of the purine ring with a CH group. This result further highlighted this region of the cyclic compound as important for the production of a full agonist response. This modification also changed the properties of the purine ring such that the N1 position was more basic with the result that the N1-ribosyl linkage was less susceptible to cleavage. This has resulted in the first compound which is stable to both chemical and enzymatic hydrolysis.

By combining the replacement of the N7 nitrogen atom with a substitution of a Br atom at the 8-position we have been able to synthesise the first hydrolytically stable antagonist of this Ca^{2+} release mechanism. These two modifications have also conferred a certain degree of hydrophobicity to this cyclic analogue enabling it to be able to traverse the cell membrane. This membrane permeable non-hydrolysable antagonist is a most important pharmacological tool.

The introduction of this thesis contains a summary diagram of the structure activity relationships of cADPR to its Ca^{2+} releasing role which were either known at the outset of this project or have been published whilst this work has been ongoing. Below is the same diagram now updated to include the significant results which have been achieved by this thesis.

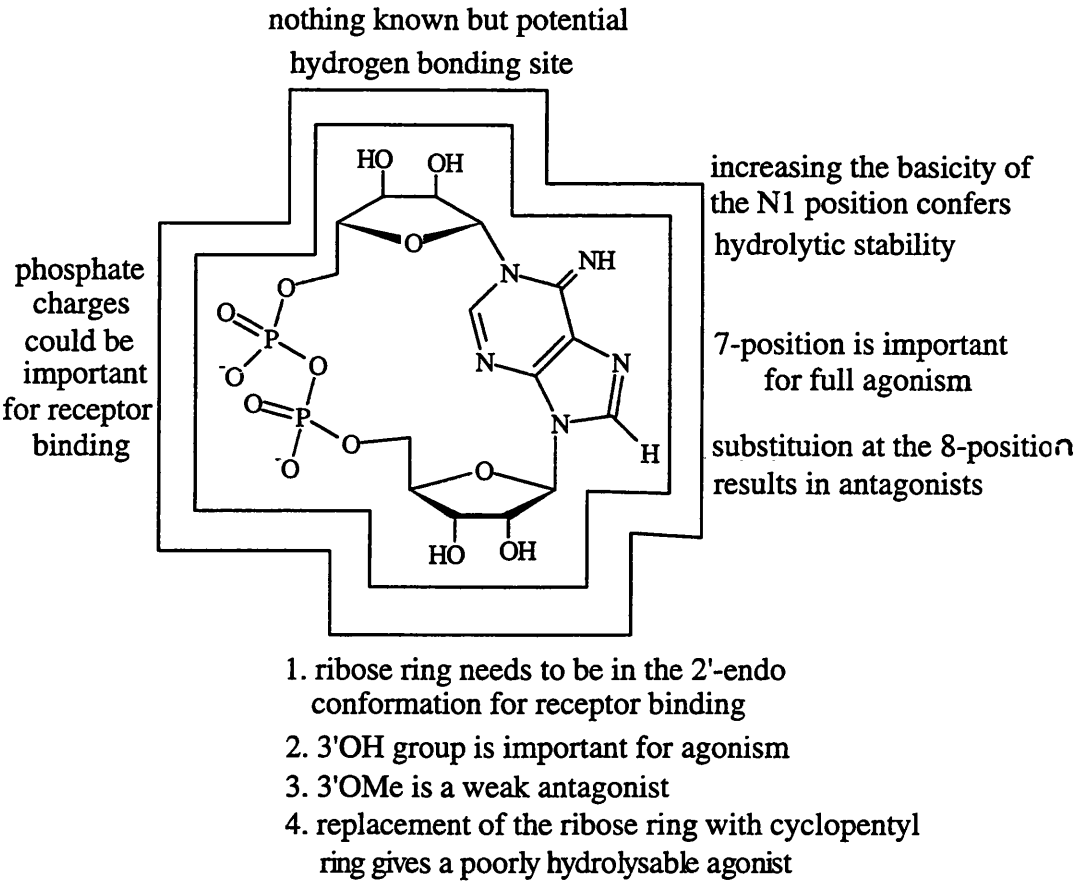


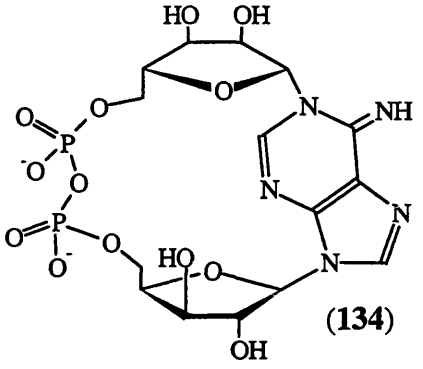
Figure 6.19 Known Structure Activity Profile for cADPR.

6.5 Further Work

As with any project there is, of course, the limitation of time. Below I wish to outline that work which I feel would be the most useful next steps in continuing this project to build upon the observations which have been made in this thesis.

(i) Synthesis of the adenosine xylose analogue of cADPR (**134**, figure 6.20A) would further investigate the hypothesis that both the ring conformation and the position of the 3'OH are important for good agonist activity. I predict that this analogue would have the correct conformation for recognition but would only have a poor agonist response. Similarly in this series the synthesis of compounds in which the 3'OH was

replaced by atoms such as fluorine would also investigate the requirement for agonist or antagonist response.



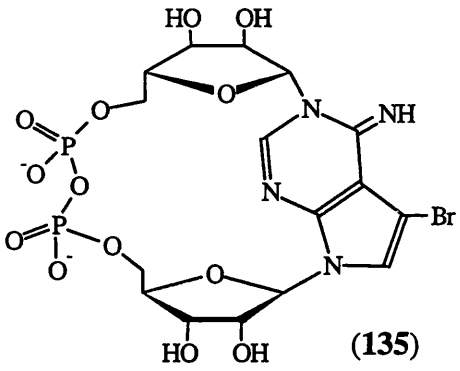
(134)

Figure 6.20A Structure of cXyloseDPR (**134**).

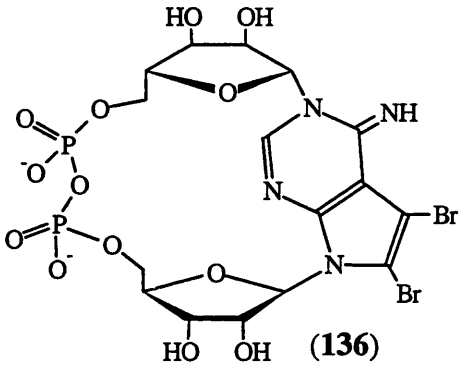
(ii) Further pharmacological work could be performed with the cAraDPR (**113**) analogue to investigate whether this ribose ring conformation was indeed important for enzymatic mediated hydrolysis. It could be that this compound might be able to bind to the hydrolase active site but not be hydrolysed and could therefore competitively inhibit this activity. This would make it an important pharmacological tool since it could be used in systems with high hydrolase activity to inhibit this action and to allow further investigation of the role of cADPR. It would also be of interest to compare the hydrolysis of cAraDPR (**113**) using cADPR hydrolase to that of CD38 to investigate any differences.

(iii) The importance of the purine ring for agonism and antagonism could further be investigated by the synthesis and testing of 7-bromo-7-deaza-cADPR (**135**) and 7-deaza-7,8-dibromo-cADPR (**136**) (figure 6.20B). If the antagonism activity was maintained, the second of these compounds would be expected to be highly membrane

permeable and therefore potentially more important than the 7-deaza-8-bromo-cADPR analogue already synthesised.



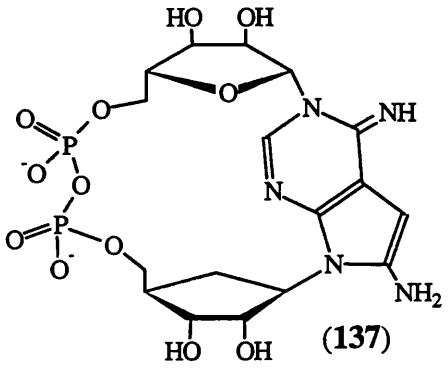
(135)



(136)

Figure 6.20B Structures of 7-Bromo-7-Deaza-cADPR (**135**) and 7-Deaza-7, 8-Dibromo cADPR (**136**).

(iv) The most potent non-hydrolysable antagonist to date would be 7-deaza-8-amino cArisDPR (**137**, figure 6.20C). Although this analogue would not have the advantage of being membrane permeable, its high potency would still make it an important pharmacological tool and therefore an important synthetic aim.



(137)

Figure 6.20C Structure of 7-Deaza-8-Amino-cArisDPR (**137**).

CHAPTER SEVEN

EXPERIMENTAL DETAIL

7.1 Materials and Methods

Thin layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminium sheets silica 60 F254, art no. 5554) with eluants as indicated. The compounds were detected using a UV lamp at 254nm. Flash chromatography refers to the procedure developed by Still *et al.*,[260] and was carried out using Sorbisil c60 silica gel.

^{1}H and ^{13}C NMR spectra were recorded on either a JEOL GX270 or 400 spectrometer. Unless otherwise stated chemical shifts were measured in parts per million (ppm) relative to residual protonated solvent. ^{31}P NMR spectra were recorded on a JEOL FX-90Q or GX400 NMR spectrometer and ^{31}P NMR chemical shifts were measured in ppm relative to external 85% H_3PO_4. Samples were recorded as 5-10% solutions in 5mm precision NMR tubes in the solvent specified except for ^{31}P NMR samples recorded on the 90FX-Q where the sample was in a nondeuterated solvent in a 5mm tube with a 10mm D_2O outer tube. In order to minimise the water peak when samples were recorded in D_2O the sample was freeze dried from dry D_2O twice before preparation. Coupling constants, J, are measured in Hertz (Hz) and the following abbreviations have been used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; ex, exchanged with D_2O. Proton assignments (where possible) and the number of protons attached to carbon atoms were established by DEPT experiments. When a sample was recorded as its triethylammonium salt the following peaks corresponding to triethylamine also appeared in the NMR spectrum:

NMR: δ_H (270MHz, D_2O); 1.24 (9H, t, J=7.3Hz, $N(CH_2\mathit{CH_3})_3$), 3.17 (6H, q, J=7.3Hz, $N(\mathit{CH_2}CH_3)_3$)

NMR: δ_C (100.4MHz, D_2O); 9.1 (CH_3), 47.5 (CH_2).

Melting points were determined using a Reichert Jung Thermo Galen Kofler block and are uncorrected.

Microanalyses were carried out at the University of Bath Microanalysis service.

Low resolution mass spectra were recorded at the University of Bath Mass Spectrometry Service using positive and negative fast atom bombardment (FAB) with 3-nitro benzyl alcohol (NBA) as a matrix carrier. High resolution mass spectra were also recorded at the University of Bath Mass Spectrometry Service. Electrospray mass spectra were recorded at the SERC Mass Spectrometry Service, University of Swansea.

Ion exchange chromatography was performed using a Pharmacia LKB Gradifrac system fitted with a 115x26mm column packed with Q-Sepharose Fast Flow resin in the bicarbonate form. The product was eluted using a linear concentration gradient of triethylammonium bicarbonate (TEAB, pH=7.8) and a flow rate of 5.0mlmin^{-1}. The column eluant was monitored by UV detection at 280nm and recorded on a chart recorder.

High performance liquid chromatography (HPLC) was performed using a Shimadzu LC-6A system connected to a Shimadzu SPD-6A UV detector operating at 259nm. The chromatographic column was a 10μm Partisil SAX anion exchange column, 120mm x .6mm, and was protected by the use of a 10μm guard column packed with the same material, 30mm x 4.6mm. The columns were isocratically eluted with 0.05M KH_2PO_4 /

5% MeOH at pH3 mobile phase with a flow rate of 1mlmin^{-1} unless otherwise stated. A sample size of 20µl was injected which had been diluted as appropriate.

References in the text to water refer to distilled water unless preceded by MilliQ. MilliQ water is high-grade deionised water and was obtained from a MilliQ Plus PF water purifying system supplied by Millipore Corp. The buffers for both ion exchange chromatography and HPLC were prepared using MilliQ water and were filtered through a Millipore filtration apparatus, pore size 0.45µm, before use. In addition the HPLC buffer was degassed by sonication for 10min.

Ultraviolet / visible (UV) spectra were recorded on a Perkin-Elmer Lambda-3 UV/Vis double beam Spectrometer using quartz cells (1cm path length) and water as the reference solvent. The spectra were recorded from 400nm-190nm and the absorbance was recorded at the λ_{max}. The sample was accurately diluted until the absorbance was less than one unit and the concentration of the sample was then calculated using the Beer-Lambert Law. Fluorescence measurements were made using a Shimadzu RF-540 spectrofluorophotometer using quartz cells. Samples were diluted as appropriate and emission and excitation ranges were as specified.

Volumes in the range 1-1000µl were measured using Gilson micropipettes with disposable plastic tips or an Hamilton syringe.

pH was measured using narrow range pH paper supplied by BDH or more accurately with a Kent EIL7020 pH meter which was standardised at pH=4.0 with 0.05M potassium

hydrogen phthalate. Conductivities were measured in $mscm^{-1}$ using a Jencons 4010 instrument.

Novel synthetic monophosphates and pyrophosphates were assayed by a quantitative adaptation of the Briggs' phosphate test.[261] A range of known aliquots of sample were pipetted into a test tube, made up to 400µl and evaporated to dryness at 200°C with each concentration being repeated in triplicate. Four drops of concentrated sulphuric acid were added to each tube which was then further heated at 200°C for 90min. The tubes were cooled to room temperature and water (200µl) was added to dissolve the residue. To each tube was then added 400µl of a solution of ammonium molybdate (2.5g) in water (20ml) and concentrated sulphuric acid (8ml), followed by 200µl of a solution of quinol (100mg) in water (20ml) with one drop of concentrated sulphuric acid, then finally 200µl of a solution of sodium sulphite (4g) in water (20ml). Each tube was heated to boiling for 10s, cooled and the volume accurately made up to 5ml with MilliQ water in a volumetric flask. The solutions were allowed to stand at room temperature for 30min and the UV absorbances recorded at 340nm. The concentration of inorganic phosphate in each sample (indicated by a blue colour) was calculated from a standard curve compiled from UV absorbance values of known concentrations of KH_2PO_4 treated as above and prepared directly before use.

Aristeromycin was kindly donated by Glaxo Wellcome, benzamide mononucleotide was donated by Professor K. Krohn (University of Paderborn) and all other chemicals were purchased from the Aldrich and Sigma chemical companies.

Dioxane refers to 1,4-dioxane; ether refers to diethyl ether; light petroleum refers to petroleum boiling in 40-60°C range.

N,N Dimethyformamide (DMF) and triethyl phosphate were dried over barium oxide, distilled under reduced pressure and stored over 4Å sieves. Pyridine was dried over potassium hydroxide pellets, distilled and then stored over potassium hydroxide pellets. Anhydrous tetrahydrofuran (THF) was obtained by distillation from sodium in the presence of benzophenone ketyl and storage was over 4Å molecular sieves. Anhydrous methanol was obtained by drying with CaH_2 followed by distillation and storage over 4Å molecular sieves. Pure and dry benzaldehyde was obtained by washing benzaldehyde with 10% Na_2CO_3 until no more CO_2 was evolved, saturated $NaSO_4$, drying over $CaCl_2$ and distillation *in vacuo*.[262] Phosphorus oxychloride was distilled under N_2 at atmospheric pressure immediately prior to use. Nucleosides were dried overnight at 60°C under reduced pressure before use.

Computer generated graphics were modelled using a Silicon Graphics Indigo cmputer and Biosym Insight and Discovery software. The structure of cADPR was fed into the computer and the following torsion angles were fixed: O′-C1′-N9-C4 64°; O″-C1″-N1-C6 167°; C2-N3-C4-N9 180°; N1-C6-C5-N7 180°. Using a CFF91 force field, a dynamics simulation was run at 1000K for 200ps saving a snap shot every ps. The energy was minimised using steepest descent and a VA09A minimiser to give the lowest energy modelled structure. This was then compared to the crystal structure of cADPR by a rigid body root mean square calculation which gave the value 0.3126 indicating the structure to be a good fit. Analogues of cADPR were then modelled in the same manner.

7.2 Synthesis of Nucleosides

7.2.1 Preparation of 2', 3'-*O*-Isopropylidene Adenosine (42)

Dry *p*-toluene sulphonic acid (TsOH, 420mg, 2.2mmol) was stirred with HPLC grade acetone (4ml) until it had dissolved. To this was added dry adenosine (534mg, 2mmol) followed by triethyl orthoformate (1.3ml) dropwise. A clear solution formed over a period of two hours which was then neutralised with dilute ammonia solution (12ml containing 0.2ml of concentrated NH_3). The volume was reduced by evaporation *in vacuo* and the residue was purified from any contaminating TsOH by recrystallisation from methanol. Several recrystallisation steps gave the crystalline product (**42**) mp 223-224°C (lit [263] 216-7°C) in 45% yield (lit [263] 87%).

NMR δ_H (400MHz, d_6DMSO, ppm); 1.31 and 1.54 (both 3H, both s, both $OCCH_3$), 3.57 (2H, m, H5'), 4.22 (1H, dt, J=7.0, 2.4Hz, H4'), 4.96 (1H, dd, J=6.2, 2.4Hz, H3'), 5.25 (1H, t, J=5.6Hz, D_2O ex, 5'OH), 5.34 (1H, dd, J=6.2, 3.1Hz, H2'), 6.12 (1H, d, J=3.1Hz, H1'), 7.36 (2H, br s, D_2O ex, NH_2), 8.15 (1H, s, H2), 8.34 (1H, s, H8),

NMR δ_C (100.4MHz, d_6DMSO, ppm); 27.3, 25.4 (CCH_3) 61.7 (C5'), 81.6 (C3'), 83.4 (C2'), 86.5 (C4'), 89.9 (C1'), 113.4 (CCH_3), 119.1 (C5), 140.0 (C8), 149.0 (C4), 152.9 (C2), 156.1 (C6),

m/z (FAB^+) 308.2 (M^++1), 135 (adenine), (FAB^-) 171 (M^+-adenine),

$C_{13}H_{17}N_5O_4$: Calculated C:50.8 H:5.53 N:22.8

Found C:50.9 H:5.64 N:22.4.

7.2.2.1 Attempted Preparation of 2', 3'-*O*-Benzylidene Adenosine (45)

To a stirred solution of dry adenosine (516mg, 1.93mmol) in dry DMF (20ml) was added dry TsOH (670mg, 3.5mmol) followed dropwise by benzaldehyde dimethylacetal (3ml, 2.1mmol). The flask was fitted with an air condenser attached to a water pump via a three-way tap and evacuated and the system was stirred with heating at 50°C overnight. The solution was cooled and the solvent removed *in vacuo*. TLC 10%MeOH/$CHCl_3$ showed no discernible product.

7.2.2.2 Attempted Preparation of 2', 3'-*O*-Benzylidene Adenosine (45)

Dry TsOH (420mg, 2.2mmol) was added to a flask containing freshly distilled benzaldehyde (5.6ml, 3.92mmol). Dry adenosine (534mg, 2mmol) was added in one portion and stirred while triethyl orthoformate (1.3ml) was added dropwise. The mixture was stirred overnight but never became a clear solution and after removal of the solvent *in vacuo* TLC 10%MeOH/$CHCl_3$ confirmed that no product had been formed.

7.2.2.3 Preparation of 2', 3'-*O*-Benzylidene Adenosine (45)

Zinc chloride (1.4g) was stirred with freshly distilled benzaldehyde (7ml) until a viscous solution formed, approximately 1h. To this was then added dry adenosine (500mg, 1.87mmol) in one portion and a clear solution was formed after a further 1h. Stirring was continued overnight and the clear solution was poured into ether (100ml) which had been

dried over K_2CO_3. After shaking the precipitated zinc chloride salt of the benzylidene product was rapidly filtered off and dissolved in ethoxyethanol (8ml). Aqueous NaOH (4.8ml of a 10% solution) was added and the mixture was set aside for 10minutes. CO_2 was bubbled into this solution until it was neutral to phenolpthalein and the precipitated inorganic salts were removed by filtration and washed thoroughly with hot ethoxyethanol. The combined filtrate and washings were evaporated to a small volume to which cold water was added to precipitate a crude product. This was purified further by recrystallisation from aqueous ethanol to give the title compound (**45**) as white crystals mp 217-220°C (lit [132] 224°C) in 35% yield (lit [132] 75%).

NMR δ_H (400 MHz, d_6DMSO, ppm); 3.46-3.60 (2H, m, H5'), 4.23-4.26 (0.5H, m, H4' exo), 4.31-4.34 (0.5H, m, H4' endo), 5.02-5.05 (1H, m, H3'), 5.12 (0.5H, br t, J=6.8Hz, D_2O ex, 5'OH), 5.23 (0.5H, br t, J=6.8Hz, D_2O ex, 5'OH), 5.42-5.51 (1H, m, H2'), 5.97 (0.5H, s, $(RO)_2$*CH* endo), 6.19 (0.5H, s, $(RO)_2$*CH* exo), 6.23-6.25 (1H, m, H1'), 7.38-7.52 (7H, m, NH_2 Ph; D_2O ex, 5H), 8.11 (1H, s, H2), 8.33 (1H, s, H8),

NMR δ_C (67.8 MHz, d_6DMSO, ppm); 61.6 (C5'), 80.8, 82.9 (C2' or C3'), 84.1, 84.6 (C2' or C3'), 86.5 (C1' or C4'), 88.1, 89.7 (C1' or C4'), 103.2, 106.8 (*C*$(RO)_2$), 119.2 (C5), 127.1, 127.2, 128.6, 128.7, 129.9, 130.0, 136.3, 136.4, (Ph), 139.9, 140.1 (C8), 149.0, 149.2 (C4), 152.9 (C2), 156.2 (C6),

m/z (FAB$^+$) 356.2 (M^++1), (FAB$^-$) 508 (M^+-1+ NBA), 354.1 (M^+-1), 134 (adenine),

$C_{17}H_{17}N_5O_4$: (M^++1) Requires 356.12805

Found 356.13470, 357.13721.

7.2.3 Synthesis of 9-[(2-Hydroxyethoxy)methyl] adenine (49)

7.2.3.1 Preparation of (2-Acetoxyethoxy) methyl bromide (54)

Freshly distilled acetyl bromide (6.5g, 53mmol) was stirred with cooling in an ice bath while 1,3-dioxolane (3.6ml, 50mmol) was added dropwise. Stirring was continued at room temperature for 30 min and the mixture purified by vacuum distillation to give a clear liquid product (**54**) bp 85-87°C at 3mmHg (lit [140] 58-60°C, 0.1mmHg) in 78% yield (lit [140] 88%). The product was used within one month of preparation since with time it went cloudy and became impure.

NMR δ_H (270 MHz $CDCl_3$, ppm); 2.10 (3H, s, $OCCH_3$), 3.87, 3.89 (2H, AB, J_{AB}=3.3Hz, $AcOCH_2\mathit{CH_2}$), 4.27, 4.29 (2H, AB, J_{AB}=3.3Hz, $AcO\mathit{CH_2}CH_2$), 5.71 (2H, s, $O\mathit{CH_2}Br$).

7.2.3.2 Preparation of 9-[(2-Acetoxyethoxy) methyl] adenine (56)

To a stirred solution of dry adenine (1.49g, 11mmol) in dry DMF (250ml) under an atmosphere of nitrogen was added lithium bis(trimethylsilyl)amide as a 1.0M solution in tetrahydrofuran (13ml, 13mmol). This was stirred at room temperature for 20min until a cloudy solution formed and was then cooled to -63°C using an acetone/CO_2 bath. (2-Acetoxy ethoxy)methyl bromide (**54**, 1.97g, 10mmol) was dissolved in dry DMF (50ml) and added dropwise to the cooled solution. The mixture was then allowed to warm to room temperature over a period of 3h and 1M $NaHCO_3$ (5ml) was added to neutralise any unreacted adenine anion. The solvent was removed *in vacuo* and the residue triturated

with $CHCl_3$ to purify the product from the majority of unreacted adenine. The product was then concentrated and purified by flash chromatography eluting with a shallow gradient of 0-5%MeOH/$CHCl_3$. Fractions with an R_f value of 0.24 in 10%MeOH/$CHCl_3$ were combined to give a white solid product (**56**) mp 146-148°C (lit [140] 156-8°C) in 25% yield (lit [140] 75%).

NMR δ_H (270 MHz, d$_6$DMSO, ppm); 1.94 (3H, s, $OCCH_3$), 3.71, 3.72 (2H, AB, J_{AB}=4.8Hz, $AcOCH_2CH_2$), 4.08, 4.09 (2H, AB, J_{AB}=4.8Hz, $AcOCH_2CH_2$), 5.58 (2H, s, OCH_2Base), 7.32 (2H, br s, D_2O ex, NH_2), 8.18 (1H, s, H2), 8.30 (1H, s, H8),

NMR δ_C (100.4 MHz, d$_6$DMSO, ppm); 20.9 ($OCCH_3$), 63.2 ($AcOCH_2CH_2$), 67.3 ($AcOCH_2CH_2$), 72.4 (OCH_2Base), 118.9 (C5), 141.5 (C8), 150.2 (C4), 153.3 (C2), 156.4 (C6), 170.6 (CO).

7.2.3.3 Preparation of 9-[(2-Hydroxyethoxy)methyl] adenine (49)

9-[(2-Acetoxyethoxy)methyl] adenine (**56**, 100mg, 0.4mmol) was dissolved in a solution of 1:1 ethanol:35% NH_3 (15ml) and heated at 50°C with stirring for 2h. The volatile materials were removed and the residue taken up in water (10ml) and washed with ethyl acetate (2x10ml). The aqueous layer was evaporated *in vacuo* to give the product which was recrystallised from methanol to give a white solid product (**49**) mp 201-3°C (lit [140] 204-6°C) in 85% yield.

NMR δ_H (270 MHz, d$_6$DMSO, ppm); 3.3-3.4 (4H, br s, $HOCH_2CH_2$, $HOCH_2CH_2$), 4.77 (1H, br s, D_2O ex, OH), 5.55 (2H, s, OCH_2Base), 7.30 (2H, br s, D_2O ex, NH_2), 8.17 (1H, s, H2), 8.27 (1H, s, H8),

NMR δ_C (100.4 MHz, d$_6$DMSO, ppm); 63.2 (AcOCH$_2$*CH$_2$*), 67.3 (AcO*CH$_2$*CH$_2$), 72.4 (O*CH$_2$*Base), 118.9 (C5), 141.5 (C8), 150.2 (C4), 153.3 (C4), 156.4 (C6),

m/z (FAB$^+$) 445.1 (M$^+$+1+ NBA), 210.1 (M$^+$+1), 121.0 (M$^+$+1-ribose),

$C_8H_{11}N_5O_2$: Calculated C:45.9 H:5.3 N:33.5

Found C:45.5 H:5.2 N:32.8.

7.2.4 Preparation of 7-Deaza-8-Bromo-Adenosine (57)

To a stirred solution of dry 7 deazaadenosine (Tubercidin, 235mgs, 0.88mmol) and dry potassium acetate (185mgs, 1.88mmol) in DMF (3.8ml) was added dropwise a solution of freshly recrystallised N-bromosuccinimide (335mgs, 1.88mmol) in DMF (1.9ml). The reaction mixture immediately turned dark red and was stirred for a further 10min. The DMF was removed by evaporation *in vacuo* and the residue dissolved in methanol and adsorbed onto silica gel. Flash chromatography was used to purify the product with elution, using a gradient of 0-10% MeOH/$CHCl_3$. Combination of the fractions containing material with a R_f of 0.07 (10%MeOH/$CHCl_3$) yielded a yellow solid product (**57**) in 40% yield (lit [150] 51.7%). This was used in the next step without further purification.

NMR δ_H (400 MHz, d$_6$DMSO, ppm); 3.48 (1H, m, H5'; D_2O ex, ABX, J_{AB}=12.2, J_{AX}=3.9Hz) 3.66 (1H, m, H5'; D_2O ex, ABX, J_{AB}=12.2, J_{AX}=3.9Hz), 3.92 (1H, dt, J=3.9, 2.4Hz, H4'), 4.14 (1H, m, H3'; D_2O ex, dd, J=5.7, 2.4Hz), 5.13 (2H, m, H2' and 3'OH; D_2O ex, dd, J=6.8, 5.7Hz, 1H), 5.31 (1H, d, J=6.6Hz, D_2O ex, 2'OH), 5.70 (1H, dd, J=5.6, 3.8Hz, D_2O ex, 5'OH), 5.84 (1H, d, J=6.8Hz, H1'), 6.80 (1H, s, H7), 7.29 (2H, br s, D_2O ex, NH_2), 8.02 (1H, s, H2),

NMR δ_C (100.4 MHz, d_6DMSO, ppm); 62.7 (C5'), 71.3 (C3'), 71.6 (C2'), 86.5 (C4'), 90.4 (C1'), 103.3 (C7), 104.3 (C5), 109.7 (C8), 149.9 (C4), 151.9 (C2), 156.9 (C6),

m/z (FAB^+) 345, 347 (M^++1), 213, 215 (M^++1-ribose).

7.2.5 Synthesis of Purine-9-β-D-2'-Deoxyribofuranoside (62)

7.2.5.1 Preparation of 3', 5'-Di-*O*-Acetyl, 2'-Deoxyadenosine (67)

Dry 2'-deoxy adenosine (0.63g, 2.5mmol) was suspended in dry pyridine (5ml) in a three necked flask under nitrogen. To this was added acetic anhydride (0.6ml, 6mmol) and 4-dimethylaminopyridine (0.05g, 0.4mmol). The solution formed was stirred at room temperature until the reaction was seen to have gone to completion by TLC with the product having an R_f value of 0.46 (10%MeOH/$CHCl_3$). MeOH (0.6ml) was then added and the volatile materials were removed by evaporation *in vacuo*. Co-evaporation of the residue with toluene (2x10ml) removed excess pyridine. The product (**67**) was purified by recrystallisation from ethanol to give a white solid mp 152-153°C (lit [264] 151-152°C) 65% yield (lit [157] 80%).

NMR δ_H (270 MHz $CDCl_3$, ppm); 2.07 and 2.11 (both 3H, both s, 3' and 5' OAc), 2.61 (1H, ddd, J=14.6, 5.6, 2.3Hz, H2'β), 2.94 (1H, ddd, J=14.6, 7.6, 6.6Hz, H2'α), 4.35-4.50 (3H, m, H4' and H5'), 5.41 (1H, m, H3'), 6.40-6.45 (3H, m, H1' and NH_2; D_2O ex, dd, J=7.6, 5.6Hz, 1H), 7.98 (1H, s, H2), 8.32 (1H, s, H8),

NMR δ_C (67.8 MHz $CDCl_3$, ppm); 20.7 (CH_3), 20.8 (CH_3), 37.4 (C2'), 63.7 (C5'), 74.3 (C3'), 82.4 (C1' or C4'), 84.5 (C1' or C4'), 119.8 (C5), 138.3 (C8), 149.4 (C4), 153.0 (C2), 155.6 (C6), 170.3 (CO), 170.4 (CO).

7.2.5.2 Preparation of 1-Pentyl Nitrite

Concentrated H_2SO_4 (17ml) was carefully added to H_2O (13ml) and the mixture left to cool. To this was added 1-pentanol (70ml) and the final solution cooled on ice.

Sodium nitrite (48g, 0.7mol) in a two neck flask was dissolved in water (190ml) by stirring and cooled to -5°C using an ice-salt bath. The 1-pentanol/H_2SO_4/H_2O mixture was added dropwise over a period of 2h during which time the temperature of the flask's contents was not allowed to rise above 0°C. When addition was complete the resulting yellow mixture was allowed to separate at room temperature for 1.5h.

The mixture was filtered to remove the precipitate and the lower aqueous layer of the filtrate was also discarded. The organic layer was washed (1x50ml of H_2O containing 1g of $NaHCO_3$ and 12.5g NaCl) and dried using Na_2SO_4. Filtration to remove the drying agent gave the product, a yellow liquid, which was purified by distillation at atmospheric pressure bp 94-96°C (lit [157] 104°C) in 92% yield. This was stored at 2°C in the dark and used within 2 weeks.

7.2.5.3 Preparation of Purine-9-β-D-3', 5'-Di-*O*-Acetyl-2'-Deoxyribofuranoside (69)

3', 5'-Di-*O*-acetyl-2'-deoxyadenosine (**67**, 0.2g, 0.6mmol) was suspended in dry THF (3ml) in a thick walled glass vessel fitted with a Teflon screw cap. 1-Pentyl nitrite (0.6ml) was then added and the reaction mixture stirred at 50°C for 48h. A second

addition of 1-pentyl nitrite (0.6ml) was then made followed by stirring at 50°C for a further 48h.

The reaction mixture was evaporated, the residue dissolved in $CHCl_3$ (20ml) and washed with 5% $NaHCO_3$ solution (2x10ml) and saturated NaCl solution (1x10ml). The organic layer was dried using $NaSO_4$, filtered and evaporated to give a dense oil which was purified by flash chromatography using 5% MeOH/$CHCl_3$. The product (**69**), which was shown to be pure by TLC (R_f 0.42, 5% MeOH/$CHCl_3$), was an oil that could not be induced to crystallise in 55% yield (lit [265] 64%).

NMR δ_H (400 MHz $CDCl_3$, ppm); 2.06 and 2.13 (both 3H, both s, 3' and 5' OAc), 2.66 (1H, ddd, J=14, 2,6.4, 2.5Hz, H2'β), 3.05 (1H, ddd, J=14.2, 7.8, 6.8Hz, H2'α), 4.31-4.43 (3H, m, H5' and H4'), 5.46 (1H, ddd, J=6.8, 3.9, 2.5Hz, H3'), 6.52 (1H, dd, J=7.8, 6.4Hz, H1'), 8.31 (1H, s, H2), 8.97 (1H, s, H8), 9.14 (1H, s, H6),

NMR δ_C (67.8 MHz $CDCl_3$, ppm); 20.6 (CH_3), 20.8 (CH_3), 37.4 (C2'), 63.7 (C5'), 74.3 (C3'), 82.5 (C1' or C4'), 84.5 (C1' or C4'), 134.5 (C5), 143.4 (C8), 148.8 (C6), 150.6 (C4), 152.5 (C2), 170.1 (CO), 170.2 (CO).

7.2.5.4 Preparation of Purine-9-β-D-2'-Deoxyribofuranoside (62)

Purine-9-β-D-3', 5'-di-*O*-acetyl-2'-deoxyribofuranoside (**69**, 96mg, 0.3mmol) was dissolved in a mixture of 1:1 ethanol:35% NH_3 (4ml) and stirred in a sealed container at 50°C for 3h. After this time the vessel was cooled on ice and TLC (5% MeOH/$CHCl_3$) showed complete conversion of the starting material to product. The volatile materials were evaporated *in vacuo* and the product dissolved in H_2O (10ml). This was washed

with ethyl acetate (2x10ml) and the aqueous layer evaporated to dryness to give the crude product (**62**) in 90% yield. This was purified by recrystallisation from EtOH to give crystalline material, mp 187-189°C, (lit [266] 192-194°C).

NMR δ_H (400 MHz d_6DMSO, ppm); 2.35 (1H, ddd, J=13.2, 6.4, 3.4Hz, H2'β), 2.78 (1H, ddd, J=13.2, 6.8, 6.4Hz, H2'α), 3.51 (1H, m, H5'), 3.61 (1H, m, H5'), 3.88 (1H, dt, J=7.8, 4.4Hz, H4'), 4.44 (1H, m, H3'), 4.97 (1H, t, J=5.4Hz, D_2O ex, 5'OH), 5.36 (1H, d, J=4.4Hz, D_2O ex, 3'OH), 6.48 (1H, dd, J=6.8, 6.4Hz, H1'), 8.81 (1H, s, H2), 8.95 (1H, s, H8), 9.18 (1H, s, H6),

NMR δ_C (100.4 MHz $CDCl_3$, ppm); 38.9 (C2'), 61.5 (C5'), 70.6 (C3'), 83.6 (C1' or C4'), 87.9 (C1' or C4'), 134.2 (C5), 145.4 (C8), 148.1 (C6), 150.6 (C4), 152.0 (C2),

m/z (FAB$^+$) 237.1 (M^++1), 117.0 (ribose+1), 213, 215 (M^++1-ribose), (FAB$^-$) 389.0 (M^+-1+ NBA), 235.0 (M^+-1), 119 (M^+-1- ribose),

$C_{10}H_{12}N_4O_3Br$:	Calculated	C:50.8 H:5.1 N:23.7
	Found	C:50.6 H:5.1 N:23.7.

7.2.6 Synthesis of 2', 3'-Isopropylidene Purine-9-β-D-Ribofuranoside (71)

7.2.6.1 Preparation of 2', 3', 5'-Tri-*O*-Acetyl Adenosine (66)

To a stirred suspension of dry adenosine (1.82g, 6.82mmol) in pyridine (15ml) under an atmosphere of nitrogen was added acetic anhydride (1.8ml, 18mmol) and dimethyl amino pyridine (0.15g, 1.2mmols). The clear solution, which formed after 2h, was stirred at room temperature overnight. After this time a precipitate was seen and TLC (10% MeOH/$CHCl_3$) showed the reaction to have gone to completion to give a product with an

R_f value of 0.46. MeOH (1ml) was then added, the reaction mixture stirred for a further 30mins and the volatile materials were removed by evaporation *in vacuo*. Co-evaporation of the residue with toluene (2x10ml) removed any excess pyridine and the product (**66**) was purified by recrystallisation from ethanol to give a white solid mp 175-177°C (lit [267] 174°C) in 90% yield.

NMR δ_H (270 MHz $CDCl_3$, ppm); 2.08, 2.11 and 2.14 (all 3H, all s, 2', 3' and 5' OAc), 4.35-4.47 (3H, m, H4' and H5'), 5.68 (1H, dd, J= 4.4, 5.3Hz, H3'), 5.93 (1H, dd, J=5.3, 5.5Hz, H2'), 6.19 (1H, d, J=5.3Hz, H1'), 6.22 (2H, br s, D_2O ex, NH_2), 7.97 (1H, s, H2), 8.35 (1H, s, H8),

NMR δ_C (100.4 MHz $CDCl_3$, ppm); 20.4 (CH_3) 20.5 (CH_3), 20.7 (CH_3), 63.1 (C5'), 70.6 (C3'), 73.1 (C2'), 80.2 (C1' or C4'), 86.1 (C1' or C4'), 119.8 (C5), 138.7 (C8), 149.7 (C4), 153.3 (C2), 155.5 (C6), 169.4 (CO), 169.6 (CO), 170.4 (CO).

7.2.6.2 Preparation of Purine-9-β-D-2', 3', 5'-Tri-*O*-Acetyl Ribofuranoside (68)

To a suspension of 2', 3', 5'-tri-*O*-acetyl adenosine (**66**, 2g, 5mmol) in dry THF (30ml) in a three-necked flask fitted with a reflux condenser and under an atmosphere of N_2 was added 1-pentyl nitrite (6ml) and the reaction mixture stirred at 50°C for 48hr. A second addition of 1-pentyl nitrite (6ml) was then made followed by stirring at 50°C for a further 48h.

The reaction mixture was evaporated, the residue dissolved in $CHCl_3$ (50ml) and washed with 5% $NaHCO_3$ solution (2x25ml) and saturated NaCl solution (1x25ml). The organic layer was dried using $MgSO_4$, filtered and evaporated to give a dense oil which was

purified by flash chromatography using 5% MeOH/$CHCl_3$. Two products were separated with R_f values of 0.69 and 0.79 (10%MeOH/$CHCl_3$). The first was an oil which could not be induced to crystallise and this was shown to be the desired product purine-9-β-D-2', 3', 5'-tri-*O*-acetyl ribofuranoside (**68**) in 65% yield. The second spot was a solid and NMR and mass spectra revealed this as the by-product 2', 3', 5'-tri-*O*-acetyl inosine (**70**) in 10% yield This material had a melting point of 226-229°C (lit [267] 236°C).

Purine-9-β-D-2', 3', 5'-tri-*O*-acetyl ribofuranoside (**68**):

NMR δ_H (270 MHz $CDCl_3$, ppm); 1.92, 1.94 and 1.99 (all 3H, all s, 2', 3' and 5' OAc), 4.24-4.33 (3H, m, H4' and 5'), 5.55 (1H, dd, J=5.1, 4.9Hz, H3'), 5.86 (1H, dd, J=4.9, 4.8Hz, H2'), 6.15 (1H, d, J=4.8Hz, H1'), 8.22 (1H, s, H2), 8.83 (1H s, H8), 9.00 (1H, s, H6),

NMR δ_C (67.8 MHz $CDCl_3$, ppm); 19.9 (CH_3), 20.0 (CH_3), 20.2 (CH_3), 62.6 (C5'), 70.1 (C3'), 72.6 (C2'), 79.9 (C1' or C4'), 86.0 (C1' or C4'), 134.1 (C5), 143.7 (C8), 148.5 (C6) 150.5 (C4), 152.3 (C2), 169.0 (CO), 169.1 (CO), 169.9 (CO),

2', 3', 5'-tri-*O*-acetyl inosine (**70**):

NMR δ_H (270 MHz $CDCl_3$, ppm); 2.05, 2.09 and 2.10 (all 3H, all s, 2', 3' and 5' OAc), 4.34-4.42 (3H, m, H4' and H5'), 5.57 (1H, dd, J=4.6, 5.5Hz, H3'), 5.84 (1H, dd, J=5.3, 5.5Hz, H2'), 6.13 (1H, d, J=5.3 Hz, H1'), 7.26 (1H, s, D_2O ex, OH), 8.01 (1H, s, H2), 8.31 (1H, s, H8),

NMR δ_C (100.4 MHz $CDCl_3$, ppm); 20.0 (CH_3), 20.3 (CH_3), 20.4 (CH_3), 62.7 (C5'), 70.2 (C3'), 73.2 (C2'), 80.2 (C1'), 86.4 (C4'), 125.1 (C5), 138.6 (C8), 145.8 (C2), 148.5 (C4), 158.6 (C6), 169.5 (CO), 169.8 (CO), 170.1 (CO),

m/z (FAB$^+$) 789.4, ($2M^+$+1), 395.2 (M^++1), 259.1 (ribose+1), (FAB$^-$) 547.1 (M^+-1+ NBA), 393.0 (M^+-1), 134.0 (M^+-1- ribose).

7.2.6.3 Preparation of Purine-9-β-D-Ribofuranoside (63)

Purine-9-β-D-2', 3', 5'-tri-*O*-acetyl ribofuranoside (**68**, 1.25g, 3.3mmol) was dissolved in a mixture of 1:1 ethanol:35% NH_3 (50ml) and stirred at 50°C for 4hr. After this time the TLC (5% MeOH/$CHCl_3$) showed complete conversion of the starting material to product. The volatile materials were evaporated *in vacuo* and the product dissolved in H_2O (50ml). This was washed with ethyl acetate (2x25ml) and the aqueous layer evaporated to dryness to give the crude product which was recrystallised from methanol to give a crystalline product mp 174-177°C (lit [268] 181-182°C) in 18% yield (lit [152] 31%).

NMR δ_H (400 MHz d_6DMSO, ppm); 3.56 (1H, m, H5'), 3.67 (1H, m, H5'), 3.97 (1H, dt, J=3.9, 3.4Hz, H4'), 4.17 (1H, dd, J=4.9, 3.4Hz, H3'), 4.61 (1H, dd, J=5.9, 4.9Hz, H2'), 5.11 (1H, t, J=5.5Hz, D_2O ex, 5'OH), 5.26 (1H, d, J=4.8Hz, D_2O ex, 3'OH), 5.55 (1H, d, J=5.9Hz, D_2O ex, 2'OH), 6.04 (1H, d, J=5.9Hz, H1'), 8.83 (1H, s, H2), 8.95 (1H, s, H8), 9.18 (1H, s, H6),

NMR δ_C (100.4 MHz, d_6DMSO, ppm); 61.5 (C5'), 70.6 (C3'), 74.0 (C2'), 86.0 (C1' or C4'), 87.8 (C1' or C4'), 134.5 (C5), 145.8 (C8), 148.6 (C6), 151.3 (C4), 152.5 (C2),

m/z (FAB$^+$) 406.1 (M^++1+ NBA), 253.1 (M^++1), 133.0 (M^++1-purine), (FAB$^-$) 405.1 (M^+-1+ NBA), 251.1 (M^+-1), 119.0 (M^+-1- ribose),

$C_{10}H_{12}N_4O_4$:	Calculated	C:47.6 H:4.76 N:22.2
	Found	C:47.2 H:4.97 N:21.7.

7.2.6.4 Preparation of 2', 3'-*O*-Isopropylidene Purine 9-β-D-Ribofuranoside (71)

Dry TsOH (105mg, 0.65mmol) was stirred with HPLC grade acetone (1ml) until it had dissolved. To this was added dry purine 9-β-D ribofuranoside (**63**, 132mg, 2mmol) followed by triethyl orthoformate (0.35ml) dropwise. A clear solution formed over a period of 1h and this was then neutralised with dilute ammonia solution (3ml containing 50μl of concentrated NH_3). The volume was reduced by evaporation *in vacuo* and the residue was purified from any contaminating TsOH by flash chromatography 5% MeOH/$CHCl_3$. Fractions containing the appropriate material with an R_f of 0.5 (5% MeOH/$CHCl_3$) were combined, the solvent removed by evaporation to give an oil (**71**) which could not be induced to crystallise in 79% yield.

NMR δ_H (270 MHz d_6DMSO, ppm); 1.27 (3H, s, CH_3), 1.5 (3H, s, CH_3), 3.72, 3.87 (2H, ABX, J_{AB}=12.5Hz, J_{AX}=1.8Hz, H5'), 4.43 (1H, m, H4'), 5.00 (1H, dd, J=6.0, 5.7Hz, H3'), 5.13 (1H, dd, J=5.7, 4.6Hz, H2'), 5.20-5.45 (1H, br s, D_2O ex, 5'OH), 5.91 (1H, d, J=4.6Hz, H1'), 8.13 (1H, s, H2), 8.85 (1H, s, H8), 9.06 (1H, s, H6),

NMR δ_C (67.8 MHz, d_6DMSO, ppm);), 25.1 (C(CH_3)$_2$), 27.4 (C(CH_3)$_2$), 62.9 (C5'), 81.4 (C3'), 83.1 (C2'), 86.2 (C4'), 93.5 (C1'), 114.3 (*C*(CH_3)$_2$), 134.5 (C5), 145.8 (C8), 148.6 (C6), 151.3 (C4), 152.5 (C2),

m/z (FAB$^+$) 293.1 (M^++1), 121.0 (M^++1-ribose), (FAB$^-$) 445.1 (M^+-1+ NBA), 291.1 (M^+-1),

$C_{13}H_{16}N_4O_4$ (M^++1): Requires 293.11716

Found 293.12556.

7.3 Synthesis of 5'-Monophosphates - the Nucleotides

All the following nucleosides were phosphorylated selectively at the 5' position using essentially the methodology developed by Yosikawa *et al.*.[179] Unfortunately no one procedure could be developed to satisfactorily phosphorylate the whole series of compounds so there are variations in the ratios of reagents, reaction time and methods of purification depending on the nucleoside being phosphorylated.

7.3.1 Preparation of Adenosine 5'-Monophosphate (19)

Dry adenosine (403mg, 1.5mmol) was suspended in triethyl phosphate (5ml) and a clear solution was obtained by heating, with swirling, in a flask fitted with a drying tube. This solution was then cooled to 0°C with an ice bath, phosphorus oxychloride ($POCl_3$, 300µl, 1.5mmol) was added dropwise and the mixture stirred for 3h at room temperature. The reaction was quenched by the addition of iced water (20ml), the mixture stirred for a further 30min and then the majority of the solvent was removed *in vacuo*. Upon the addition of MilliQ water (0.5ml) a small amount of white solid product precipitated which was further encouraged by the addition of ice cold acetone (5ml). The precipitation was allowed to stand at 0°C overnight, the supernatant was carefully removed and the solid product was further purified by ion exchange chromatography. The fractions containing UV active material, those which eluted between 150 and 220mM TEAB, were pooled, evaporated *in vacuo* and excess TEAB was coevaporated with MeOH. The pure material (**19**) was obtained in 39% yield as the triethylammonium salt as calculated by UV and was

confirmed to be the desired product by comparison with authentic AMP from Sigma using HPLC, NMR and UV.

NMR: δ_H (270MHz, D_2O); 3.96 (2H, m, H5'), 4.32 (1H, m, H4'), 4.47 (1H, dd, J=3.7, 4.6Hz, H3'), 4.75 (1H, m, H2'), 6.06 (1H, d, J=5.7Hz, H1'), 8.12 (1H, s, H2), 8.54 (1H, s, H8), the H2' peak was partially obscured by the water peak at 4.8ppm,

NMR: δ_P (161.7MHz, D_2O); 3.66ppm, broadens when proton coupled,

NMR: δ_C (67.8MHz, D_2O) 63.1 (C5'), 70.4 (C3'), 74.2 (C2'), 84.5 (C1'), 86.5 (d, J_{CP}=7.7Hz, C4'), 118.2 (C5), 139.9 (C8), 148.7 (C4), 152.4 (C2), 155.2(C6),

UV: λ_{max} 259nm, ε=15.3x10^3 $M^{-1}cm^{-1}$,[269]

HPLC retention time: 2.15min.

7.3.2 Preparation of Adenine 9-β-D-Arabinofuranoside 5'-Monophosphate (72)

Dry adenine 9-β-D-arabinoribofuranoside (177mg, 0.66mmol) was suspended in triethyl phosphate (2ml) and dissolved by heating to 50°C for 15min. The solution was cooled to 0°C, $POCl_3$ was added (140µl, 0.70mmol) and the reaction mixture was stirred overnight at 4°C. The reaction was quenched with ice (10g) and the material was purified by ion exchange chromatography. The crude product which eluted between 140 and 200mM TEAB was achieved in 58% yield as calculated by UV analysis but was still shown to be contaminated with inorganic phosphate by phosphorus NMR. A second product was also obtained from the ion exchange column which eluted between 210 and 250mM TEAB in 4.5% yield and was material which had been phosphorylated at either the 2' or 3' hydroxyl.

The major product was loaded onto a column of activated charcoal (3cmx1.5cm) which had previously been washed with MilliQ water. Inorganic phosphate was removed by washing the column with water (100ml), the product was then eluted with aqueous ethanolic ammonia (H_2O:EtOH:NH_3, 24:25:1, 600ml) and its recovery was monitored by UV. The fractions were collected and the solvent removed *in vacuo* to yield the ammonium salt of the desired product with 66% recovery from the charcoal column. The product was then dissolved in MilliQ water (2ml) and MeOH (0.75ml) and was precipitated using acetone (27.5ml). The precipitate was collected by centrifugation, washed with acetone and dried to give the title compound (**72**).

Title compound (**72**):

NMR: δ_H (270MHz, D_2O); 4.0-4.3 (3H, m, H4', H5'), 4.38 (1H, dd, J=6.6, 6.4Hz, H3'), 4.59 (0.7H, dd, J=6.6, 5.5Hz, H2'), 5.23 (0.3H, m, H2'), 6.15 (0.3H, d, J=5.5Hz, H1'), 6.42 (0.7H, d, J=5.5Hz, H1'), 8.34 (0.3H, s, H2) 8.38 (1H, s, H8), 8.53 (0.7H, s, H2),

δ_P (161.7MHz, D_2O); 3.10ppm, broadens when proton coupled,

δ_C (100.4MHz, D_2O) 63.1 (C5'), 68.9 (C3'), 80.6, 80.8 (C2'), 86.4 (d, J_{CP}=7.4Hz, C4'), 89.5 (C1'), 123.3 (C5), 148.5, 149.0 (C4 or C8), 149.8, 150.1 (C4 or C8), 153.5 (C2), 155.1(C6),

UV: λ_{max} 259nm, ε=15.3x10^3 $M^{-1}cm^{-1}$,[270]

HPLC retention time: 2.21min,

m/z (FAB^+)102 (triethylamine); (FAB^-) 494 (M^+ +NBA-2), 346 (M^+-1).

Minor product (**138**):

δ_P (161.7MHz, D_2O); -0.54ppm, (d, J_{PH}= 8.2Hz),

UV: λ_{max} 259nm, ε= 15.3x10^3 $M^{-1}cm^{-1}$(as for AMP),

HPLC retention time: 2.36min,

m/z (FAB^+) 364 (M^+-2+H_2O); (FAB^-) 492 (M^++NBA-4).

7.3.3 Preparation of Adenine-9-[β-(2α,3α-di-hydroxy-4β(hydroxymethyl)-cyclopentyl)] 5'-Monophosphate (73)

To a cooled solution of dry Aristeromycin (193mg, 0.725mmol) suspended in triethyl phosphate (2ml) as described above, was added $POCl_3$ (150µl, 0.75mmol) and the reaction stirred over night at 4°C. The reaction was quenched using ice (10g) and purified by ion exchange chromatography. When the fractions containing the product, which eluted between 150 and 210mM TEAB, had been combined and the solvent removed the desired product (**73**) was achieved in 56% yield. However phosphorus NMR showed this to be contaminated with inorganic phosphate. This contaminant was removed using a charcoal column as described above with 72% recovery. The ammonium salt was precipitated and the product collected by centrifugation and dried to give adenine-9-[β-(2α, 3α-di-hydroxy-4β(hydroxymethyl)-cyclopentyl)] 5'-monophosphate (**73**).

NMR: δ_H (270MHz, D_2O); 1.91-1.98 (1H, m, H6'), 2.37-2.43 (1H, m, H4'), 2.45-2.53 (1H, m, H6'), 3.97-4.04 (2H, m, H5'), 4.12 (1H, dd, J=2.8, 5.7Hz, H3'), 4.49 (1H, dd, J=5.7, 9.3Hz, H2'), 4.85-4.93 (1H, m, H1'), 8.37 (1H, s, H2), 8.46 (1H, s, H8),

δ_P (161.7MHz, D_2O); 0.81ppm, (t, J_{PH}= 5.0Hz),

δ_C (100.4MHz, D_2O) 32.9 (C6'), 47.5 (d, J_{CP}=7.4Hz, C4'), 64.9 (C1'), 71.3 (C5'), 76.5 (C2' or C3'), 80.2 (C2' or C3'), 123.1 (C5), 148.0 (C8), 148.7 (C4), 153.6 (C2), 154.4 (C6),

UV: λ_{max} 259nm, ε=15.3x10^3 $M^{-1}cm^{-1}$ [271],

HPLC retention time: 2.55min,

m/z (FAB$^-$) 646.4 ($2M^+$), 344.1 (M^+-2), 195.0 (M^+-adenine-H_2O), 97.0 ($PO_4H_2^{2-}$).

7.3.4 Preparation of 2', 3'-*O*-Isopropylidene Adenosine 5'-Monophosphate (74)

To a cooled solution of dry 2', 3'-*O*-isopropylidene adenosine (**42**, 200mg, 0.65mmol) suspended in triethyl phosphate (2ml) was added to $POCl_3$ (140µl, 0.70mmol) and the reaction stirred over night also at 4°C. It was quenched by the addition of ice (10g) and purified by ion exchange chromatography. When the fractions containing the product, which eluted between 120 and 180mM TEAB, had been combined and the solvent removed the desired product (**74**) had been achieved in 47% yield. This was further purified from inorganic phosphate using a charcoal column with 88% recovery of the desired product. The ammonium salt was precipitated as above, the product collected by centrifugation and dried to give 2', 3'-isopropylidene adenosine 5'-monophosphate (**74**).

NMR: δ_H (400MHz, D_2O); 1.43, 1.66 (both 3H, both s, C(CH_3)$_2$), 3.98 (2H, m, H5'), 4.60 (1H, m, H4'), 5.04 (1H, dd, J=2.0, 5.9Hz, H3'), 5.35 (1H, dd, J=3.4, 5.9Hz, H2'), 6.19 (1H, d, J=3.4Hz, H1'), 8.12 (1H, s, H2), 8.42 (1H, s, H8),

δ_P (161.7MHz, D_2O); 3.51ppm, broadens when proton coupled,

δ_C (100.4MHz, D_2O); 27.2, 29.0 (C(CH_3)$_2$), 67.3 (d, J_{CP}=3.7Hz, C5'), 84.3 (C2' or C3'), 86.7 (C2' or C3'), 87.8 (d, J_{CP}=9.2Hz, C4'), 92.7 (C1'), 117.6 ($C(CH_3)_2$), 121.1 (C5), 143.0 (C8), 151.4 (C4), 155.5 (C2), 158.1 (C6),

UV: λ_{max} 259nm, ε= 15.2x10^3 M^{-1}cm^{-1},[187]

HPLC retention time: 2.98min,

m/z (FAB$^+$) 388.1 (M^++1), 102.0 (triethylamine); (FAB$^-$) 773.0 ($2M^+$-1), 386.1 (M^+-1).

7.3.5 Preparation of 2', 3'-*O*-Benzylidene Adenosine 5'-Monophosphate (75)

To a cooled solution of dry 2', 3'-*O*-benzylidene adenosine (**45**, 153mg, 0.43mmol) suspended in triethyl phosphate (2ml) was added $POCl_3$ (150µl, 0.75mmol) and the reaction stirred overnight at 4°C. The reaction was quenched by addition of ice (50g) followed by stirring at room temperature for 1h. The solution was adjusted to pH=8.0 using 1M TEAB and diluted to 500ml.

The material was loaded onto an Amberlite XAD-4 column (15cmx1.5cm), the column washed with water (200ml) and the product then recovered using a gradient elution of aqueous MeOH (0-100% MeOH over 720ml). The fractions containing the product were identified using UV, combined and the solvent removed *in vacuo*. Phosphorus NMR showed there to be no contaminating inorganic phosphate but UV analysis showed the recovery from the column to be a disappointing 14%. The material was contaminated with unreacted nucleoside (30% by HPLC) so the compound as further purified by ion exchange chromatography and the product eluted (**75**) between 250 and 300mM TEAB. The solvent was removed in vacuo, excess TEAB co-evaporated using MeOH to give 2′, 3′-O-benzylidene adenosine 5′-monophosphate (**75**).

NMR: δ_H (400MHz, D_2O); 3.83-3.92 (2H, m, H5'), 4.44-4.45 (0.3H, m, H4'), 5.07-5.10 (1H, m, H3'), 5.30-5.33 (1H, m, H2'), 5.93 (0.3H, s, *CH*$(RO)_2$), 6.08 (0.7H, s, *CH*$(RO)_2$), 6.15 (0.7H, d, J=2.9Hz, H1'), 6.20 (0.3H, s, H1'), 7.28-7.47 (5H, m, Ph), 7.98 (0.3H, s, H8), 7.99 (0.7H, s,H2), 8.21 (0.7H, s, H8) 8.27 (0.3H, s, H2), the remaining H4' peak is obscured by the water signal at 4.8ppm,

δ_P (161.7MHz, D_2O); -0.649ppm, (septet, J_{PH}=8.06Hz),

NMR δ_C (67.8 MHz, d_6DMSO, ppm); 64.1 (C5'), 80.1, 82.6 (C2' or C3'), 83.3, 84.1 (C2' orC3'), 87.9(C1' or C4'), 89.7 (C1' or C4'), 103.5, 107.1 ($C(RO)_2$), 126.5, 126.7, 128.4, 128.5, 130.1, 130.3, 134.4 (Ph), 139.9 (C8), 148.3 (C4), 152.4 (C2), 155.1 (C6), (C5) peak was not seen,

UV: λ_{max} 259nm, ε=12.16x10^3 M^{-1}cm^{-1},

HPLC retention time: 3.65min with the HPLC system running at 2mlmin^{-1},

m/z (FAB$^+$) 436 (M$^+$+1); 434 (M$^+$-1).

7.3.6.1 Attempted Preparation of Acycloadenosine Monophosphate (76)

$POCl_3$ (45µl, 0.32mmol) was slowly added to a cooled stirred suspension of dry 9-[(2-hydroxyethoxy)methyl] adenine (**49**, 10mg, 48µmol) in *m*-cresol (1ml). After stirring at 5h at 0°C an aliquot (10µl) was removed, quenched with water (1ml) and analysed by HPLC (starting material 1.77min, product 2.5min). The trace showed the reaction to be only 35% complete and the reaction was not continued any further.

7.3.6.2 Preparation of Acycloadenosine Monophosphate (76)

Dry 9-[(2-hydroxyethoxy)methyl] adenine (**49**, 50mg, 0.24mmol) in triethylphosphate (2ml) was heated to 50°C for 15min, cooled to 0°C and $POCl_3$ (75µl, 0.53mmol) was added slowly. The reaction was warmed to room temperature and the phosphorylation monitored by HPLC as above. When the reaction was complete (1.5h) it was quenched by the addition of iced water (5g) and the product purified by ion exchange chromatography. The fractions containing the product, which eluted between 130 and

190mM TEAB, were combined and the solvent removed *in vacuo* to give the title compound (**76**) in 55% yield. ^{31}P NMR showed the product to be contaminated with inorganic phosphate. The product was purified using a charcoal column as previously described with 95% recovery followed by precipitation using methanol : acetone 1:10 (2ml) to give the pure product (**76**).

NMR δ_H (400 MHz, D_2O, ppm); 3.57-3.60 (2H, m, $POCH_2CH_2$), 3.74-3.78(2H, m, $POCH_2CH_2$), 5.66 (2H, s, OCH_2Base), 8.16 (1H, s, H2), 8.26 (1H, s, H8),

δ_P (161.7MHz, D_2O); 1.48ppm, broadens when proton coupled,

NMR δ_C (100.4 MHz, D_2O, ppm); 64.5 (d, J_{CP}=5.5Hz, $POCH_2CH_2$), 69.8 (d, J_{CP}=9.2Hz, $POCH_2CH_2$), 73.9 (OCH_2Base), 119.2 (C5), 143.4 (C8), 149.7 (C4), 153.6 (C2), 156.3 (C6),

UV: λ_{max} 259nm, ε=15.3x10^3 $M^{-1}cm^{-1}$ (as for adenosine),

HPLC retention time: 2.5min,

m/z (FAB^+) 290.1 (M^++1), 255 (M^+-2H_2O+2); (FAB^-) 577 ($2M^+$-1).

7.3.7 Preparation of 7-Deazaadenosine 5'-Monophosphate (77)

A mixture of dry 7-deazaadenosine (Tubercidin, 82mg, 308µmol) and triethyl phosphate (1ml) was heated at 50°C for 15min, cooled to 0°C and $POCl_3$ (50µl, 535µmol) added dropwise. A clear solution was seen after 5min and this was stirred at 4°C for 18h. Iced water (10ml) was added and the mixture was stirred for a further 2h. The product was purified by ion-exchange chromatography and fractions containing the product, which eluted between 190mM and 220mM were pooled, evaporated *in vacuo* and excess TEAB

was coevaporated with MeOH. The product was further purified using a charcoal column to give the product (**77**) as its ammonium salt in 48% yield as calculated by UV analysis.

NMR: δ_H (270MHz, D_2O); 3.94 (2H, dd, J=3.1, 4.8Hz, H5'), 4.19 (1H, dt, J=3.1, 5.2Hz, H4'), 4.30 (1H, dd, J=3.1, 5.0Hz, H3'), 4.49 (1H, dd, J=5.0, 6.7Hz, H2'), 6.14 (1H, d, J=6.7Hz, H1'), 6.72 (1H, d, J=4.0Hz, H7), 7.54 (1H, d, J=4.0Hz, H8), 8.08 (1H, s, H2),

δ_P (161.7MHz, D_2O); 0.02ppm (td, J_{PH}=2.4, 4.9Hz),

δ_C (100.4MHz, D_2O); 67.3 (C5'), 73.5 (C3'), 77.1 (C2'), 86.8 (d, J_{CP}=9.2Hz, C4'), 89.2 (C1'), 101.7 (C5), 105.5 (C7), 127.2(C8), 145.3 (C4), 150.4 (C2), 153.9 (C6),

UV: λ_{max}=266nm, ε=12.2x$10^3M^{-1}cm^{-1}$,[272]

HPLC retention time 1.98min.

7.3.8 Preparation of 8-Bromo-Adenosine 5'-Monophosphate (78)

To a cooled solution of 8-bromo-adenosine (50mg, 144µmol) suspended in triethyl phosphate (1ml) was added $POCl_3$ (50µl, 535µmol) and the reaction was monitored by HPLC (starting material 2.04min, product 2.31min). When the material was seen to be over 90% phosphorylated (2.5h) the reaction was quenched by addition of 3:1 water : pyridine (2ml) and the mixture diluted and purified by ion exchange chromatography. The fractions containing the title compound, which eluted between 200 and 240mM TEAB, were pooled and the solvent removed *in vacuo* to give the product (**78**) in 53% yield. This was contaminated with a 10 fold impurity of inorganic phosphate as indicated by phosphorus NMR but no further purification was attempted.

NMR: δ_H (400MHz, D_2O); 4.10 (2H, m, H5'), 4.25 (1H, m, H4'), 4.56 (1H, dd, J=4.9, 5.4Hz, H3'), 5.19 (1H, dd, J=5.7, 5.8Hz, H2'), 6.07 (1H, d, J=5.8Hz, H1'), 8.14 (1H, s, H2),

δ_P (161.7MHz, D_2O); 3.40ppm, broadens when proton coupled,

δ_C (100.4MHz, D_2O); 65.3 (d, J_{CP}=3.7Hz, C5'), 70.5 (C3'), 71.8 (C2'), 84.4 (d, J_{CP}=9.2Hz, C4'), 89.2 (C1'), 118.3 (C5), 139.9 (C8), 150.7 (C4), 153.6 (C2), 154.9 (C6),

UV: λ_{max} 263nm, ε=15.1x10^3 $M^{-1}cm^{-1}$ [189],

HPLC retention time: 2.31min.

7.3 9 Preparation of 7-Deaza-8-Bromo-Adenosine 5'-Monophosphate (79)

To a cooled solution of dry 7-deaza-8-bromo-adenosine (**57**, 100mg, 290μmol) suspended in triethyl phosphate (2ml) was added $POCl_3$ (100μl, 500μmol) and the reaction was monitored by HPLC (starting material 1.74min, product 2.15min). After 90min cooled 1:9 pyridine: water (10ml) was added to the above followed by stirring for a further 30min. This product (**79**) was purified by ion exchange chromatography and the fractions containing the product, which eluted between 310 and 370mM TEAB, were pooled evaporated *in vacuo* to give the pure product (**79**) in 44% yield.

NMR: δ_H (400MHz, D_2O) 4.08-4.15 (3H, m, H4' and H5'), 4.53 (1H, dd, J=5.7, 5.8Hz, H3'), 5.07 (1H, dd, J=5.3, 5.8Hz, H2'), 6.04 (1H, d, J=5.3Hz, H1'), 6.42 (1H, s, H7), 7.92 (1H, s, H2),

δ_P (161.7MHz, D_2O); 2.35ppm, broadens when proton coupled,

δ_C (100.4MHz, D_2O); 65.2 (d, J_{CP}=3.7Hz, C5'), 70.3 (C3'), 72.1 (C2'), 83.6 (d, J_{CP}=7.4Hz, C4'), 89.8 (C1'), 104.0 (C5), 105.2 (C7), 110.4 (C8), 149.9 (C4), 150.0 (C2), 154.5 (C6),

UV: λ_{max} 276nm, ε=13.34x10^3 M^{-1}cm^{-1},

HPLC retention time: product 2.15min,

m/z (FAB$^+$) 425, 427 (M$^+$+1); (FAB$^-$) 423, 425 (M$^+$-2), 341 (M$^+$-Br-3).

7.3.10 Preparation of Inosine 5'-Monophosphate (80)

Dry inosine (268mg, 1mmol) was suspended in triethyl phosphate (3ml) and dissolved by heating with a heat gun (5min). The solution was cooled to 0°C, $POCl_3$ (200µl, 1.0mmol) added, and the reaction stirred at 4°C for a further 4h. Addition of ice (10g) with further stirring for 30min quenched the reaction and any volatile material was removed by evaporation *in vacuo*. To the remaining residue (approximately 1ml) was added MilliQ water (1 drop) and the precipitate was separated by centrifugation and purified by ion exchange chromatography. Fractions which eluted between 180 and 250mM TEAB were pooled, the solvent removed and the title compound (**80**) was obtained in 31.7% yield.

NMR: δ_H (400MHz, D_2O); 4.08 (2H, m, H5'), 4.34 (1H, m, H4'), 4.46 (1H, dd, J=4.4, 4.7Hz, H3'), 4.69 (1H, dd, J=4.4, 5.9Hz, H2'), 6.05 (1H, d, J=5.9Hz, H1'), 8.12 (1H, s, H2), 8.39 (1H, s, H8),

δ_P (161.7MHz, D_2O); 1.59ppm (t, J_{PH}=3.3Hz),

δ_C (100.4MHz, D_2O) 65.1 (d, J_{CP}=5.5Hz, C5'), 71.2 (C3'), 75.4 (C2'), 85.0 (d, J_{CP}=9.2Hz, C4'), 88.3 (C1'), 124.4 (C5), 140.5 (C8), 147.0 (C2), 149.5 (C4), 159.2 (C6),

UV: λ_{max} 248nm, ε=12.3x10^3 M^{-1}cm^{-1},[273]

HPLC retention time: 1.93min,

m/z (FAB$^+$) 349.1 (M$^+$+1), 102 (triethylamine); (FAB$^-$) 694.9 (2M$^+$-1), 500.1 (M$^+$+NBA-1) 347.1 (M$^+$-1).

7.3.11 Preparation of Purine 9-β-D-2'-Deoxy Ribofuranoside 5'-Monophosphate (81)

To a cooled solution of dry purine 9-β-D-2'-deoxy ribofuranoside (**62**, 171mg, 0.73mmol) suspended in triethyl phosphate (3ml) was slowly added $POCl_3$ (136µl, 0.97mmol). The reaction was quenched after 24h at 0°C using 1:5 pyridine:water (6ml) and the mixture was purified by ion exchange chromatography. The fractions which eluted between 50 and 110mM TEAB, were combined and the solvent removed *in vacuo* to give a product in 68%. The material was further purified from inorganic phosphate using a charcoal column with a poor recovery of material (37%). The ^{1}H coupled ^{31}P NMR spectrum was a doublet indicating that this major product was in fact material phosphorylated at the 3' hydroxyl.

NMR δ_H (400 MHz D_2O, ppm); 2.66-2.72 (1H, m, H2'), 2.84-2.98 (1H, m, H2'), 3.39, 3.42 (2H, ABX, J=7.2, 1.2Hz, H5'), 4.37-4.39 (1H,m, H4'), 4.89-4.94 (1H, m, H3'), 6.48 (1H, dd, J=7.0, 6.4Hz, H1'), 8.62 (1H, s, H2), 8.82 (1H, s, H8), 8.99 (1H, s, H6),

δ_P (161.7MHz, D_2O); -0.649 ppm (d, J_{PH}=7.6Hz),

UV: λ_{max}=262nm ε=7.35x10^3 $M^{-1}cm^{-1}$ (as for **88**),

HPLC retention time: 3.45min.

7.3.12 Preparation of Purine 9-β-D-Ribofuranoside 5'-Monophosphate(88)

7.3.12.1 Preparation of Purine 9 β-D-2', 3'-*O*-Isopropylidene Ribofuranoside 5'-(dicyanoethyl)-Monophosphate (86)

A mixture of dry bis(cyanoethoxy)(diisopropylamino)phosphine [274] (162mg, 0.6mmol), dry CH_2Cl_2 (2ml) and 1*H*-tetrazole (63mg, 0.9mmol) was stirred at room temperature for 15min whereupon a solution of purine 9-β-D-2', 3'-isopropylidene ribofuranoside (**71**, 70mg, 240μmol) in dry CH_2Cl_2 (2ml) was added and stirring continued for a further 30min. ^{31}P NMR spectroscopy showed a phosphite peak at δ_P at 149.7ppm, tetrazole activated phosphite peak at δ_P 128.7ppm and a phosphate peak at -1.56ppm. The system was cooled to -78°C and MCPBA (210mg, 1.2mmol) was added with stirring for 10 min whilst warming to room temperature. The mixture was extracted with EtOAc (50ml) and this organic extract was washed with aqueous 10% Na_2SO_3 (50ml), saturated Na_2CO_3 (2x50ml) and saturated NaCl (50ml). The EtOAc layer was dried using $MgSO_4$, filtered and concentrated to give a pale yellow solid which was purified by flash chromatography (elutent $CHCl_3$ then 10% MeOH/$CHCl_3$) to give the title compound as an oil (**86**, 100mg, 87%).

NMR: δ_H (270MHz, D_2O); 1.4, 1.58 (both 3H, both s, $C(CH_3)_2$), 2.65, 2.69 (4H, AB, J_{AB}=6.1Hz, CH_2CN), 4.10-4.32 (6H, m, OCH_2, H5'), 4.45 (1H, m, H4'), 5.09 (1H, dd, J=3.5, 6.2Hz, H3'), 5.40 (1H, dd, J=2.3, 6.2Hz, H2'), 6.18 (1H, d, J=2.3Hz, H1'), 8.22 (1H, s, H2), 8.95 (1H, s, H8), 9.10 (1H, s, H6),

δ_P (161.7MHz, D_2O); -2.49ppm (septet, J_{PH}=7.6Hz),

δ_C (67.8MHz, D_2O); 19.5, 19.6 (CH_2CN), 27.0, 25.2 ($C(CH_3)_2$), 62.3, 62.4 (OCH_2), 67.5 (C5'), 83.9 (C2' or C3'), 85.0 (C2' or C3'), (C1'), 86.5 (C4'), 134.6 (C5), 144.4 (C8), 149.0 (C6), 150.5 (C4), 152.8(C2),

m/z (FAB^+) 479 (M^++1), 359 (M^+-purine); (FAB^-) 631 (M^+-1+NBA), 424 (M^+-cyanoethyl).

7.3.12.2 Preparation of Purine-9-β-D-2', 3'-*O*-Isopropylidene Ribofuranoside 5'-Monophosphate (87)

Purine 9 β-D-2', 3'-*O*-isopropylidene ribofuranoside 5'-(di-cyanoethyl)-monophosphate (100mg, 210μmol) was dissolved in concentrated ammonia solution (5ml) and stirred at room temperature overnight. This material was purified by ion exchange chromatography and eluted between 150 and 170mM TEAB. The fractions containing the product were evaporated to dryness in vacuo to give the title compound (**87**) in 29% yield.

NMR: δ_H (400MHz, D_2O); 1.30, 1.53 (both 3H, boths, CH_3), 3.87, (2H, m, H5′), 4.54 (1H, m, H4′), 5.06 (1H, m, H3′), 5.31 (1H, dd, J=3.1, 5.7Hz, H2′), 6.26 (1H, d, J=3.1Hz, H1′), 8.64 (1H, s, H2), 8.78 (1H, s, H8), 8.96 (1H, s, H6),

NMR: δ_P (161.7MHz, D_2O); singlet at 1.11ppm which broadens with ^1H coupling,

UV: λ_{max} 262nm ε=7. 35x$10^3M^{-1}cm^{-1}$ (as for **88**),

HPLC retention time: 5.17min.

7.3.11.3 Purine-9-β-D- Ribofuranoside 5'-Monophosphate (88)

H^+ Dowex resin (0.5ml) was washed copiously with MilliQ water (200ml) and this was then added to a solution of purine-9-β-D-2', 3'-*O*-isopropylidene ribofuranoside 5'-monophosphate triethyl ammonium salt (**87**, 60μmol) in MilliQ water (2ml). The mixture was stirred and the removal of the isopropylidene was monitored by HPLC with the retention time shifting from 5.1min to 3.1min as the reaction proceeded. When the reaction was complete (5h at room temperature, 30min at 50°C) the resin was removed by filtration, washed with MilliQ water to recover the monophosphate product which was then in turn purified by ion exchange chromatography. Fractions which eluted between 170 and 200mM TEAB were combined, the solvent removed in vacuo to give the title compound (**88**) in 72% yield.

NMR: δ_H (400MHz, D_2O); 3.96, (2H, m, H5'), 4.25 (1H, m, H4'), 4.36 (1H, m, H3'), 6.14 (1H, d, J=5.5Hz, H1'), 8.72 (1H, s, H2), 8.80 (1H, s, H8), 8.98 (1H, s, H6), the H2' peak is obscured by the water peak at 4.8ppm,

NMR: δ_P (161.7MHz, D_2O); singlet at 1.15ppm which broadens with ^1H coupling,

NMR: δ_C (100.4MHz, D_2O); 64.9 (d, J=3.7Hz, C5'), 71.4 (C2' or C3'), 75.3 (C2' or C3'), 85.2 (d, J=9.2Hz, C4'), 88.1 (C1'), 134.3 (C5), 146.1 (C8), 148.7 (C6), 151.8(C4), 152.9 (C2),

UV: λ_{max} 262nm, ε=7.35 $M^{-1}cm^{-1}$,[275]

HPLC retention time: 3.1min,

m/z (FAB^-) 663 ($2M^+$-1), 331 (M^+-1).

7.4 Synthesis of Analogues of Nicotinamide Adenine Dinucleotide

Nicotinamide adenine dinucleotide analogues were synthesised by coupling the monophosphate nucleotide to nicotinamide mononucleotide. Two different methods were used to achieve this, depending upon the monophosphate, and they are described in detail below for the parent compound. The variations for each individual coupling reaction are then described more briefly.

7.4.1 Preparation of Nicotinamide Adenine Dinucleotide (8, NAD^+)

7.4.1.1 Dicyclohexyl Carbodiimide Coupling Method

To a stirred solution of AMP (**19**, triethylammonium salt, 240μmol) and NMN (**18**, free acid, 50mg, 150μmol) dissolved in MilliQ water (2.5ml) was added pyridine (10ml) and dicyclohexyl carbodiimide (DCC, 2g). The mixture was stirred at room temperature for 7 days after which the reaction was quenched by addition of MilliQ water (100ml) and standing at 4°C for 2h. The mixture was filtered to remove the by-product dicyclohexyl urea (DCU) and the filtrate washed with $CHCl_3$ (3x50ml). The product was purified by ion exchange chromatography using a gradient elution of TEAB 0-250mM over 720ml. The fractions containing the product, which eluted between 80 and 130mM TEAB, were pooled, the solvent removed *in vacuo* and any excess TEAB was removed by coevaporation of the sample with MeOH. When compared to authentic NAD^+ this sample was confirmed to be the title compound (**8**) in 20% yield as caluclated by UV analysis.

7.4.1.2 Diphenylchorophosphate Coupling Method

7.4.1.2.1 Preparation of 2', 3'-Di-*O*-acetyl NMN (99)

A solution of NMN (**18**, free acid, 100mg, 300μmol) in H_2O (0.6ml) was added dropwise to a rapidly stirred mixture of acetic anhydride (6ml) in dry pyridine (10ml). This was stirred at room temperature for 2h and then overnight at 4°C. The solvent was removed *in vacuo,* keeping the bath temperature below 30°C, and the residue was stirred with aqueous pyridine (H_2O:pyridine, 20:1, 1ml) for 0.5h to destroy any mixed anhydride and any excess of acetic anhydride. After removal of the solvent, the resulting residue was suspended in MeOH (5ml) and tri-*n*-octylamine (140μl, 300μmol) was added. The mixture was stirred for 0.5h, the MeOH was evaporated, and the crude product (**99**, tri-*n*-octylammonium salt) was dried three times by coevaporation of any excess water with dry DMF and used directly in the next step.

7.4.1.2.2 Activation of AMP with Diphenylchlorophosphate (100)

AMP (**19**, free acid, 174mg, 500μmol) was dissolved in anhydrous MeOH (5ml). To this was added 1 equivalent of tri-*n*-octylamine (220μl) and the mixture was stirred until a clear solution was seen, (approximately 0.5h). The solvent was evaporated and the resulting mono-tri-*n*-octylammonium salt dried by evaporation of three volumes of dry DMF (2ml). The residue was dissolved in a mixture of dry DMF (2ml) and dry dioxane (3ml) and diphenylchlorophosphate (DPPC,167μl, 835μmol) was added, followed by tri-*n*-butylamine (167μl, 500μmol). An homogenous solution formed after a few minutes

and this was stirred at room temperature for a further 2h. After this time ^{31}P NMR spectrum (36.2MHz, D_2O in a 10mm outer tube) showed no peak at approximately 0ppm corresponding to AMP. The solvent was evaporated *in vacuo* and the residue was shaken with cold, dry ether (2x5ml) which removed any unreacted diphenyl chlorophosphate and gave a sticky white solid. The majority of the ether was decanted and any residual ether was removed by evaporation.

7.4.1.2.3 Coupling of Activated AMP (100) to 2', 3'-Di-*O*-acetyl NMN (99)

To a solution of activated AMP (**100**) dissolved in dry DMF (0.75ml) was added 2', 3'-di-*O*-acetyl NMN (**99**) also dissolved in DMF (0.75ml) followed immediately by dry pyridine (1.5ml) and the reaction was stirred at room temperature for 18h. After this time the solvent was evaporated, any traces of pyridine removed by coevaporation with MeOH and the product was deacetylated *in situ* by dissolving the residue in ice cold methanolic ammonia (1:1 ratio of MeOH with 35% ammonia solution with a total volume of 5ml) and stirring for 6h at 4°C. Again the solvent was removed *in vacuo* and the residue was purified by ion-exchange chromatography using a gradient elution of 0-250mM TEAB over 720ml. The fractions containing the product, which eluted between 80 and 130mM TEAB, were pooled, evaporated *in vacuo* and excess TEAB was coevaporated with MeOH. The title compound (**8**) was obtained as the triethylammonium salt in 24% yield over the three steps, as quantified by UV measurements.

NMR: δ_H (270MHz, D_2O); 4.22 (2H, m, $2H_A5'$), 4.37 (2H, m, $2H_N5'$), 4.42-4.52 (5H, m, H_N2', H_N3', H_A3', H_A4', H_N4'), 4.74 (1H, m, H_A2'), 5.99 (1H, d, J=6.0Hz, H_A1'), 6.06

(1H, d, J=5.3Hz, H_N1'), 8.09 (1H, s, H_A2), 8.14 (1H, dd, J=7.3, 6.2Hz, H_N5), 8.39 (1H, s, H_A8), 8.81 (1H, d, J=7.3Hz, H_N4), 9.13 (1H, d, J=6.2Hz, H_N6), 9.31 (1H, s, H_N2),

δ_P (161.7MHz, D_2O); -10.49, -10.94ppm (2d, J_{PP}=23.4Hz),

δ_C (100.4MHz, D_2O); 64.6 (C_A5'), 65.0 (C_N5'), 70.0 (C_A3'), 70.3 (C_N3'), 73.6 (C_A2'), 77.2 (C_N2'), 83.4 (d, J=8.9Hz, C_A4'), 86.3 (C_A1'), 86.6 (d, J=7.7Hz, C_N4'), 99.6 (C_N1'), 118.1 (C_A5), 128.3 (C_N4), 133.3 (C_N5), 139.4 (C_A8), 139.6 (C_N3), 142.1 (C_N6), 145.4 (C_N2), 148.6 (C_A4), 152.4 (C_A2), 155.0 (C_A6), 164.8 (CO),

UV: λ_{max}=259nm, ε=17.3x$10^3M^{-1}cm^{-1}$,[276]

HPLC retention time 2.48min.

7.4.2 Preparation of 2'$_N$, 3'$_N$-Di-*O*-acetyl Nicotinamide Adenine Dinucleotide (101, AcONAD$^+$)

AMP (**19**, free acid, 110μmol) was changed to the tri-*n*-octylammonium salt as previously described. The material was dried and dissolved in dry DMF (0.6ml) and dry dioxane (1.0ml) to which was added DPPC (50μl, 250μmol) and tri-*n*-butylamine (50μl, 180μmol). After stirring for 2h the solvent was evaporated and the residue triturated with cold, dry ether. NMN (**18**, 50mg, 150μmol) was diacetylated as described, dissolved in dry DMF (0.75ml) added directly to the activated AMP (**100**) in dry pyridine (0.75ml) and stirred at room temperature for 18h. After this time the solvent was evaporated and the residue was purified by ion-exchange chromatography. The fractions containing the product, which eluted between 100 and 110mM TEAB, were pooled, the pH adjusted to 6.0 using 1M HCl and evaporated *in vacuo*. The compound was further purified using a

reverse phase silica column and eluting with 1% aqueous MeOH. This gave the title compound (**101**) in 8.7% yield as a clear glassy solid.

NMR: δ_H (400MHz, D_2O); 2.73, 2.89 (both 3H, both s, $COCH_3$), 4.03-4.61 (9H, m, H_A2', H_N3', H_A3', H_A4', H_N4', $2H_A5'$, $2H_N5'$), 5.23 (1H, m, H_N2'), 5.92 (1H, d, J=5.8Hz, H_A1'), 5.97 (1H, d, J=6.4Hz, H_N1'), 8.06 (1H, s, H_A2), 8.14 (1H, m, H_N5), 8.32 (1H, s, H_A8), 8.76 (1H, d, J=7.9Hz, H_N4), 9.03 (1H, d, J=6.4Hz, H_N6), 9.22 (1H, s, H_N2),

δ_P (36.2MHz, D_2O); -11.31, -11.95ppm (2d, J_{PP}=14.9Hz),

UV: λ_{max}=259nm, ε=17.3x$10^3M^{-1}cm^{-1}$ (as for NAD^+),

HPLC retention time 2.72 min,

m/z (FAB^+) 749 (M^++1), 663 (M^+-2$COCH_3$+1), 614 (M^+-adenine+1), 530 (M^+-2$COCH_3$-adenine-1), 447 (M^+-adenosine-H_2O+1).

7.4.3 Preparation of Arabino$_A$ Nicotinamide Adenine Dinucleotide (103, NAraD$^+$)

To a stirred solution of adenine-9-β-D- arabinofuranoside 5'-monophosphate (**72**, 240μmol) and NMN (**19**, 50mg, 150μmol) in MilliQ water (2.5ml) was added pyridine (10ml) and an excess of DCC (2g). The reaction was stirred at room temperature for 7 days, quenched by the addition of MilliQ water (100ml), kept at 4°C for 2h, filtered and extracted with $CHCl_3$ (3x50ml). The material was purified by ion exchange chromatography and the fractions containing the product, which eluted between 70-90mM TEAB, were combined, the solvent removed *in vacuo* and excess TEAB removed by coevaporation with MeOH to yield the title compound (**103**) in 10% yield.

NMR: δ_H (270MHz, D_2O); 4.13-4.4.57 (8H, m, H_N3', H_A3', H_A4', H_N4', $2H_A5'$, $2H_N5'$), 6.05 (1H, d, J=4.2Hz, H_A1'), 6.27 (1H, d, J=5.7Hz, H_N1'), 8.13 (1H, s, H_A2), 8.19 (1H, m,

H_N5), 8.34 (1H, s, H_A8), 8.83 (1H, d, J=8.1Hz, H_N4), 9.17 (1H, d, J=5.7Hz, H_N6), 9.31 (1H, s, H_N2); H_A2' and H_N2' were obscured by the water peak at 4.8ppm,

δ_P (161.7MHz, D_2O); -11.4, -11.8ppm (2d, J_{PP}=20.8Hz),

UV: λ_{max}=259nm, ε=17.3x$10^3 M^{-1}cm^{-1}$ (as for NAD^+),

HPLC retention time 2.32min,

m/z (FAB^-) 663 (M^+-1), 648 (M^+-H_2O+2).

7.4.4 Preparation of 2'α, 3'α-Di-Hydroxy-4'β(hydroxymethyl)-cyclopentyl$_A$ Nicotinamide Adenine Dinucleotide (104, NArisD$^+$)

Aristeromycin 5'-monophosphate (**73**, free acid, 60μmol) was changed to the mono tri-*n*-octylammonium salt as previously described. The material was dried and dissolved in dry DMF (0.2ml) and dry dioxane (0.4ml) to which was added DPPC (15μl, 75μmol) and tri-*n*-butylamine (15μl, 56μmol). After stirring for 2h the solvent was evaporated and the residue triturated with cold, dry ether. NMN (**18**, free acid, 12mg, 25μmol) was diacetylated as previously described, dissolved in dry DMF (0.2ml) and added directly to the activated aristeromycin 5'-monophosphate in dry pyridine (0.2ml) and stirred at room temperature for 18h. After this time the solvent was evaporated and the product was deacetylated using 1ml of 50% methanolic ammonia as previously described. The solvent was removed *in vacuo* and the residue was purified by ion-exchange chromatography. The fractions containing the product, which eluted between 60 and 90mM TEAB, were pooled, evaporated *in vacuo* and excess TEAB was coevaporated with MeOH to give the title compound in 19% yield.

NMR: δ_H (400MHz, D_2O); 1.7-1.9 (2H, m, H_A6'), 2.3-2.4 (1H, m, H_A4') 4.22-4.80 (10H, m, H_N2', H_A2', H_N3', H_A3', H_N4', $2H_A5'$, $2H_N5'$), 5.11-5.14 (1H, m, H_A1'), 6.06 (1H, d, J=5.5Hz, H_N1'), 8.01 (1H, s, H_A8), 8.13 (1H, m, H_N5), 8.18 (1H, s, H_A2), 8.83 (1H, d, J=8.2Hz, H_N4), 9.15 (1H, d, J=6.4Hz, H_N6), 9.34 (1H, s, H_N2),

δ_P (161.7MHz, D_2O); -11.0, -11.8ppm (2d, J_{PP}=21.8Hz),

UV: λ_{max}=259nm, ε=17.3x$10^3M^{-1}cm^{-1}$ (as for NAD^+),

HPLC retention time 2.51min,

m/z (FAB^-) 658 (M^+-4), 619 (M^+-CONH), 538 (M^+-nicotinamide-2), 385 (M^+-aristeromycin-NH_2), 234 (nicotinamide ribose-H_2O-2).

7.4.5 Preparation of $2'_A$, $3'_A$-*O*-Isopropylidene Nicotinamide Adenine Dinucleotide (105, NAcetD$^+$)

To a stirred solution of 2', 3'-*O*-isopropylidene adenosine 5'-monophosphate (**74**, 240μmol) and NMN (**18**, 50mg, 150μmol) dissolved in MilliQ water (2.5ml) was added pyridine (10ml) and an excess of DCC (2g). The reaction was stirred at room temperature for 7 days and quenched by the addition of MilliQ water (100ml), kept at 4°C for 2h, filtered and extracted with $CHCl_3$ (3x50ml). The material was purified by ion exchange chromatography, the fractions which eluted between 50 and 80mM TEAB combined, the solvent removed *in vacuo* and excess TEAB removed by coevaporation with MeOH and to yield two products which could not be separated. These were the title compound (**105**) in 14.3% yield and the by-product 2', 3'-*O*-isopropylidene adenosine (5'-OMe) monophosphate (**112**) in 10.1% yield.

Title compound (**105**):

NMR: δ_H (400MHz, D_2O); 1.31 and 1.52 (both 3H, both s, C(CH_3)$_2$), 4.03-4.53 (8H, m, H_N2', H_N3', H_A4', H_N4', 2H_A5', 2H_N5'), 5.04 (1H, dd, J=5.8, 2.6Hz, H_A3'), 5.32 (1H, dd, J=5.8, 2.8Hz, H_A2'), 6.04 (1H, d, J=5.8Hz, H_N1'), 6.12 (1H, d, J=2.8Hz, H_A1'), 8.07 (1H, s, H_A2), 8.15 (1H, dd, J=7.9, 5.8Hz, H_N5), 8.20 (1H, s, H_A8), 8.83 (1H, d, J=7.9Hz, H_N4), 9.16 (1H, d, J=5.8Hz, H_N6), 9.38 (1H, s, H_N2),

δ_P (161.7MHz, D_2O); -11.8ppm multiplet,

UV: λ_{max}=259nm, ε=17.3x10^{3}M^{-1}cm^{-1} (as for NAD$^+$),

HPLC retention time 4.18min,

m/z (FAB$^-$) 580 (M$^+$-nicotinamide+2), 553 (M$^+$-adenine-H_2O+1).

by-product (**112**):

NMR: δ_H (400MHz, D_2O); 1.31 and 1.52 (both 3H, both s, C(CH_3)$_2$), 3.24 (3H, d, J=10.7Hz, PO*Me*), 3.88 (2H, m, 2H_A5'), 4.51 (1H, m, H_A4'), 5.05 (1H, m, H_A3'), 5.34 (1H, dd, J=6.1, 2.7Hz, H_A2'), 6.15 (1H, d, J=2.7Hz, H_A1'), 8.10 (1H, s, H_A2), 8.22 (1H, s, H_A8),

δ_P (161.7MHz, D_2O); 1.51ppm,

UV: λ_{max}=259nm, ε=15.3x10^{3}M^{-1}cm^{-1} (as for AMP),

HPLC retention time 3.38min,

m/z (FAB$^+$) 402 (M$^+$+1); (FAB$^-$) 400 (M$^+$-1).

7.4.6 Preparation of Acyclo$_A$ Nicotinamide Adenine Dinucleotide (106, NAcycloD$^+$)

To a stirred solution of acycloadenosine monophosphate (**76**, 14μmol) and a five fold excess of NMN (**18**, 15mg, 45μmol) in MilliQ water (0.3ml) was added pyridine (1.2ml) and an excess of DCC (0.25g). The reaction was stirred at room temperature for 7 days, quenched by the addition of MilliQ water (25ml) kept at 4°C for 2h, filtered and extracted with $CHCl_3$ (3x10ml). The material was purified by ion exchange chromatography and the fractions containing the product, which eluted between 70 and 90mM TEAB, were combined, the solvent removed *in vacuo* and excess TEAB removed by coevaporation with MeOH to yield the title compound (**106**) in 12% yield.

NMR: δ_H (270MHz, D_2O); 3.81 (2H, m, $POCH_2CH_2$), 4.07 (2H, m, $POCH_2CH_2$), 4.21-4.75 (5H, m, H_N2', H_N3', H_N4', $2H_N5'$), 5.66 (2H, s, OCH_2Base), 6.11 (1H, d, J=5.5Hz, H_N1'), 8.18-8.26 (3H, m, H_A2, H_A8, H_N5), 8.89 (1H, d, J=6.9Hz, H_N4), 9.20 (1H, d, J=6.2Hz, H_N6), 9.36 (1H, s, H_N2),

δ_P (100.4MHz, D_2O); -10.9, -10.6ppm (2d, J_{PP}=18.5Hz),

UV: λ_{max}=259nm, ε=17.3x10^{3}M^{-1}cm^{-1} (as for NAD$^+$),

HPLC retention time 3.1min.

7.4.7 Preparation of 7-Deaza Nicotinamide Adenine Dinucleotide (107, 7-Deaza-NAD$^+$)

7-Deazaadenosine 5'-monophosphate (**77**, free acid, 72μmol) was changed to the mono-tri-*n*-octylammonium salt as previously described. The material was dried and dissolved

in dry DMF (0.3ml) and dry dioxane (0.5ml) to which was added DPPC (20µl, 100µmol) and tri-*n*-butylamine (20µl, 75µmol). After stirring for 2h the solvent was evaporated and the residue triturated with cold, dry ether. NMN (**18**, 25mg, 50µmol) was diacetylated as previously described, dissolved in dry DMF (0.3ml) and added directly to the activated 7-deazaadenosine 5'-monophosphate in dry DMF (0.2ml) and dry pyridine (0.5ml) and stirred at room temperature for 18h. After this time the solvent was evaporated and the product was deacetylated using 1ml of 50% methanolic ammonia as previously described. The solvent was removed *in vacuo* and the residue was purified by ion-exchange chromatography. The fractions containing the product, which eluted between 120 and 140mM TEAB, were pooled, evaporated *in vacuo* and excess TEAB was coevaporated with MeOH to give the title compound (**107**) in 38% yield.

NMR: δ_H (270MHz, D_2O); 4.0-4.1 (4H, m, $2H_A5'$, $2H_N5'$), 4.19 (1H, m, H_A3'), 4.25 (1H, m, H_N3'), 4.29 (1H, m, H_A4'), 4.38 (1H, dd, J=5.3, 4.9Hz, H_A2'), 4.42 (1H, m, H_N2'), 4.46 (1H, t, J=5.7Hz, H_N4'), 6.11 (1H, d, J=5.3Hz, H_A1'), 6.16 (1H, d, J=6.4Hz, H_N1'), 6.65 (1H, d, J=3.6Hz, H_A5), 7.53 (1H, d, J=3.6Hz, H_A8), 8.09 (1H, s, H_A2), 8.16 (1H, dd, J=8.1, 6.4Hz, H_N7), 8.78 (1H, d, J=8.1Hz, H_N4), 9.15 (1H, d, J=6.4Hz, H_N6), 9.33 (1H, s, H_N2),

δ_P (161.7MHz, D_2O); -11.8, -11.4ppm (2d, J_{PP}=21.8Hz),

δ_C (100.4MHz, D_2O); 65.8 (d, J=3.7Hz, C_A5'), 66.5 (d, J=5.6Hz, C_N5'), 71.3 (C_A3'), 71.6 (C_N3'), 74.9 (C_A2'), 78.5 (C_N2'), 84.5 (d, J=9.2Hz, C_A4'), 87.2 (C_A1'), 87.9 (d, J=9.2Hz, C_N4'), 100.9 (C_N1'), 103.0 (C_A5), 103.4 (C_A7), 124.6 (C_A8), 129.5 (C_N4), 134.4 (C_N3), 140.8 (C_N5), 143.3 (C_N6), 146.6 (C_N2), 149.1 (C_A4), 152.4 (C_A2), 155.0 (C_A6), 166.2 (CO),

UV: λ_{max}=266nm, $\varepsilon=12.7x10^3M^{-1}cm^{-1}$,[277]

HPLC retention time 2.19min,

m/z (electrospray) 662 (M^++1), (FAB$^-$) 660 (M^+-1).

7.4.8 Preparation of 7-Deaza-8-Bromo-Nicotinamide Adenine Dinucleotide (108, 7-Deaza-8-Bromo- NAD$^+$)

To a solution of 7-deaza-8-bromo-adenosine 5′-monophosphate (**79**, 94μmol) and NMN (**18**, 20mg, 60μmol) in MilliQ water (1ml) was added pyridine (4ml) and an excess of DCC (0.5g) and the reaction was stirred at room temperature for 7 days. The reaction was quenched by the addition of MilliQ water (50ml), the mixture kept at 4°C for 2h, filtered and extracted with $CHCl_3$ (3x20ml). The material was purified by ion exchange chromatography and the fractions containing the product, which eluted between 90 and 120mM TEAB, were combined, the solvent removed *in vacuo* and excess TEAB removed by coevaporation with MeOH to yield the title compound (**108**) in 27.7% yield.

NMR: δ_H (400MHz, D_2O); 4.03-4.32 (8H, m, H_N2', H_N3', H_A4', H_N4', $2H_A5'$ $2H_N5'$), 4.56 (1H, dd, J=5.8, 5.3Hz, H_A3'), 5.16 (1H, dd, J=5.9, 5.8Hz, H_A2'), 5.99 (1H, d, J=4.4Hz, H_N1'), 6.06 (1H, d, J=5.9Hz, H_A1'), 6.60 (1H, s, H_A7), 8.05 (1H, s, H_A2), 8.14 (1H, dd, J=8.3, 6.4Hz, H_N5), 8.73 (1H, d, J=8.3Hz, H_N4), 9.09 (1H, d, J=6.4Hz, H_N6), 9.25 (1H, s, H_N2),

δ_P (161.7MHz, D_2O); -10.5, -10.9ppm (2d, J_{PP}=20.1Hz),

δ_C (100.4MHz, D_2O); 65.7 (d, J=3.7Hz, C_A5'), 66.5 (C_N5'), 70.0 (C_A3'), 71.4 (C_N3'), 71.9 (C_A2'), 78.5 (C_N2'), 83.5 (d, J=9.2Hz, C_A4'), 87.8 (d, J=9.2Hz, C_N4'), 89.8 (C_A1'), 100.9 (C_N1'), 104.3 (C_A5), 105.0 (C_A7), 110.5 (C_A8), 129.6 (C_N4), 134.2 (C_N3), 140.6 (C_N5), 143.1 (C_N6), 146.3 (C_N2), 150.4 (C_A2), 150.7 (C_A4), 155.5 (C_A6), 166.1 (CO),

UV: λ_{max}=270nm, ε=13.3x$10^3M^{-1}cm^{-1}$,

HPLC retention time 2.16min,

m/z (electrospray$^+$) 741, 743 (M^++1); (electrospray$^-$) 739, 741 (M^+-1).

7.4.9 Preparation of Nicotinamide Hypoxanthine Dinuceotide (109, NID$^+$)

Inosine 5'-monophosphate (**80**, free acid, 42μmol) was converted to the mono-tri-*n*-octylamine (1equiv, 20μl) as previously described. The material was dried, dissolved in dry DMF (0.3ml) and dry dioxane (0.5ml) and DPPC (35μl, 170μmol) and tri-*n*-butylamine (35μl, 125μmol) were added. After stirring for 2h the solvent was evaporated and the residue triturated with cold, dry ether. NMN (**18**, 20mg, 40μmol) was diacetylated as previously described, dissolved in dry DMF (0.3ml) and added directly to the activated inosine 5'-monophosphate in dry pyridine (0.3ml) and stirred at room temperature for 18h. After this time the solvent was evaporated and the product was deacetylated using 1ml of 50% methanolic ammonia as previously described. The solvent was removed *in vacuo* and the residue was purified by ion-exchange chromatography. The fractions containing the product, which eluted between 120 and 170mM, were pooled, evaporated *in vacuo* and excess TEAB was coevaporated with MeOH to give the title compound (**109**) in 27% yield.

NMR: δ_H (270MHz, D_2O); 4.21 (2H, m, $2H_I5'$ or $2H_N5'$), 4.35 (2H, m, $2H_I5'$ or $2H_N5'$), 4.43-4.54 (5H, m, H_N2' or H_I2', H_N3', H_I3', H_I4', H_N4'), 4.70 (1H, m, H_N2' or H_I2'), 6.06 (1H, d, J=5.3Hz, H_I1'), 6.13 (1H, d, J=5.5Hz, H_N1'), 8.18 (1H, s, H_I2), 8.24 (1H, dd, J=7.9, 6.2Hz, H_N5), 8.48 (1H, s, H_I8), 8.89 (1H, d, J=7.9Hz, H_N4), 9.23 (1H, d, J=6.2Hz, H_N6), 9.37 (1H, s, H_N2); were obscured by the water peak,

δ_P (161.7MHz, D_2O); -11.8, -11.5ppm (2d, J_{PP}=19.8Hz),

UV: λ_{max}=249nm, ε=14.7x$10^3M^{-1}cm^{-1}$ [278]

HPLC retention time 3.16min.

7.4.10 Preparation of Nicotinamide Purine Dinucleotide (110, NPD^+)

To a solution of purine-9-β-D-ribofuranoside 5'-monophosphate (**88**, 40μmol) and NMN (**18**, free acid, 15mg, 40μmol) in MilliQ water (0.45ml) was added pyridine (1.8ml) and an excess of DCC (0.34g) and the reaction was stirred at room temperature for 7 days. The reaction was quenched by the addition of MilliQ water (50ml), kept at 4°C for 2h, filtered and extracted with $CHCl_3$ (3x20ml). The material was purified by ion exchange chromatography and the fractions containing the product, which eluted between 70 and 90mM TEAB, were combined, the solvent removed *in vacuo* and excess TEAB removed by coevaporation with MeOH to yield the title compound in 75% yield.

NMR: δ_H (400MHz, D_2O); 4.08-4.40 (8H, m, H_N3', H_A3', H_A4', H_N4', $2H_A5'$, $2H_N5'$), 5.96 (1H, d, J=5.5Hz, H_A1'), 6.10 (1H, d, J=5.8Hz, H_N1'), 8.13 (1H, dd, J=7.6, 6.1Hz, H_N5), 8.66 (1H, s, H_A2), 8.76 (1H, d, J=7.6Hz, H_N4), 8.79 (1H, s, H_A8), 8.98 (1H, s, H_A6), 9.09 (1H, d, J=6.1Hz, H_N6), 9.24 (1H, s, H_N2); H_N2', H_A2' were obscured by the water peak,

δ_P (161.7MHz, D_2O); -11.8, -11.5ppm (2d, J_{PP}=19.6Hz),

UV: λ_{max}=262nm, ε=11.3x$10^3M^{-1}cm^{-1}$,[279]

HPLC retention time 3.14min.

7.4.11 Preparation of Nicotinamide Benzamide Dinucleotide (111, NBD^+)

To a solution of benzamide mononucleotide (**126**, triethylammonium salt, 245μmol) and NMN (**18**, free acid, 50mg, 150μmol) in MilliQ water (2.5ml) was added pyridine (13.2ml) and an excess of DCC (2g) and the reaction was stirred at room temperature for 7 days. The coupling was quenched by the addition of MilliQ water (100ml), kept at 4°C for 2h, filtered and extracted with $CHCl_3$ (3x50ml). The material was purified by ion exchange chromatography and the fractions containing the product, which eluted between 80 and 100mM TEAB, were combined, the solvent removed *in vacuo* and excess TEAB removed by coevaporation with MeOH to yield the title compound (**111**) in 12.4% yield.

NMR: δ_H (400MHz, D_2O); 3.91-4.43 (11H, m, H_B1', H_B2', H_N2', H_N3', H_B3', H_B4', H_N4', $2H_B5'$, $2H_N5'$), 5.99 (1H, d, J=5.5Hz, H_N1'), 7.38 (1H, dd, J=7.6, 7.6Hz, H_B5), 7.52 (1H, d, J=7.6Hz, H_B6), 7.63 (1H, d, J=7.6Hz, H_B4), 7.70 (1H, s, H_B2), 8.10 (1H, dd, J=8.2, 6.1Hz, H_N5), 8.73 (1H, d, J=8.2Hz, H_N4), 9.10 (1H, d, J=6.1Hz, H_N6), 9.24 (1H, s, H_N2),

δ_P (161.7MHz, D_2O); -10.46, -10.79ppm (2d, J_{PP}=20Hz),

UV: λ_{max}=264nm, ε=2.0x$10^3M^{-1}cm^{-1}$,

HPLC retention time 3.02min,

m/z (electrospray$^+$) 648 (M^++2), 237 (nicotinamide ribose -OH).

7.5 Synthesis of Analogues of Cyclic Adenosine Diphosphate Ribose

The NAD^+ analogues were cyclised enzymatically using the general method outlined below. ADP-ribosyl cyclase was obtained by purification from *Aplysia* slugs as described [32] and used crude. The concentration of protein in the enzyme was determined by a co-worker in this laboratory to be $10mgml^{-1}$ using the method of Bradford [280] and the specific activity was known to be $1200unitmg^{-1}$.[32]

7.5.1 Preparation of Cyclic Adenosine Diphosphate Ribose (7, cADPR)

A final volume of 2.5ml of a 1.5mM solution of NAD^+ (**8**) in 25mM HEPES buffer at pH 6.8 was incubated with 10µl of crude *Aplysia* ADP-ribosyl cyclase at room temperature. The reaction was monitored by HPLC and was quenched by a twenty fold dilution of the reaction mixture with water when the peak attributable to the cADPR was no longer seen to be increasing, approximately 30min. The desired product was isolated from a small amount of unreacted starting material and the by-product nicotinamide using ion-exchange chromatography eluting with 0-250mM TEAB over 720mls. The product (**8**, cADPR) which eluted between 80 and 110mM TEAB, was evaporated to dryness *in vacuo* keeping the water bath temperature below 30°C. Excess TEAB was removed by evaporation with MeOH and the cyclic compound (**8**) was isolated as the glassy triethylammonium salt in 56% yield as quantified by UV and stored at -70°C.

NMR δ_H (400MHz, D_2O) 3.89-3.93 (1H, m, H_A5'), 3.98-4.01 (1H, m, H_R5'), 4.24-4.27 (2H, m, H_A4', H_R5'), 4.34-4.35 (2H, m, H_R3', H_A5'), 5.24 (1H, dd, J=5.2, 5.5Hz, H_A2'), 5.95

(1H, d, J=5.5Hz, H_A1'), 6.03 (1H, d, J=4.0Hz, H_R1'), 8.27 (1H, s, H_A8), 8.89 (1H, s, H_A2); other protons are obscured by the water peak at 4.8ppm,

δ_P (161.7MHz, D2O) -10.93, -11.27ppm (2d, J_{PP}=13.9Hz),

UV: λ_{max}=257nm, ε=14.3x10^{3}M^{-1}cm^{-1},[27]

HPLC: retention time 4.2min.

7.5.2 Preparation of Cyclic Adenine 9-β-D-Arabino Ribofuranoside Diphosphate Ribose (113, cAraDPR)

NAraD$^+$ (**103**, triethylammonium salt, 7.5μmol)was cyclised as described (section 7.5.1) and the product cAraDPR (**113**), which eluted between 150 and 170mM TEAB, was evaporated to dryness *in vacuo* keeping the water bath temperature below 30°C. Excess TEAB was removed by evaporation with MeOH and the cyclic compound was isolated as the glassy triethylammonium salt in 54% yield as quantified by UV and stored at -70°C.

NMR δ_H (400MHz, D_2O) 3.89-3.93 (2H, m, $2H_A5'$), 3.98-4.01 (1H, m, H_R5'), 4.24-4.27 (2H, m, H_A4', H_R5'), 4.34-4.35 (1H, m, H_R3'), 5.17 (1H, dd, J=8.07, 8.2Hz, H_A3'), 6.03 (1H, d, J=4.0Hz, H_R1'), 6.24 (1H, d, J=7.3Hz, H_A1'), 8.20 (1H, s, H_A8), 8.91 (1H, s, H_A2); other protons are obscured by the water peak at 4.8ppm,

δ_P (161.7MHz, D2O) -10.23, -11.76ppm (2m),

UV: λ_{max}=257nm, ε=14.3x10^{3}M^{-1}cm^{-1} (as for cADPR),

HPLC: retention time 4.34min,

m/z (electrospray$^+$) 542 (M^++1); (electrospray$^-$) 541 (M^+), 540 (M^+-1), 539 (M^+-2).

7.5.3 Preparation of Cyclic Aristeromycin Diphosphate Ribose (114, cArisDPR)

NArisD$^+$ (**104**, triethylammonium salt, 3.2μmol) was cyclised as described (section 7.5.1) and the product cArisDPR (**114**), which eluted between 110 and 120mM TEAB, was evaporated to dryness *in vacuo* keeping the water bath temperature below 30°C. Excess TEAB was removed by evaporation with MeOH and the cyclic compound was isolated as the glassy triethylammonium salt in 71% yield as quantified by UV and stored at -70°C.

NMR δ_H (400MHz, D_2O) 2.2-2.5 (3H, m, H_A4', H_A6'), 3.84-3.90 (1H, m, H_A5'), 3.99-4.02 (1H, m, H_R5'), 4.14 (1H, m, H_R5'), 4.34-4.35 (2H, m, H_R3', H_A5'), 4.96-4.99 (1H, m, H_A1'), 5.2-5.3 (1H, m, H_A2'), 6.06 (1H, d, J=3.4Hz, H_R1'), 8.14 (1H, s, H_A8), 8.93 (1H, s, H_A2); other protons are obscured by the water peak at 4.8ppm,

δ_P (161.7MHz, D2O) -10.23, -11.76ppm (2m),

UV: λ_{max}=260nm, ε=14.3x10^{3}M^{-1}cm^{-1} (as for cADPR),

HPLC: retention time 4.6min,

m/z (electrospray$^+$) 541 (M^++2), 540 (M^++1), (electrospray$^-$) 539 (M^+), 538 (M^+-1), 537 (M^+-2), 365 (M^+-$P_2O_7H_2$).

7.5.4 Preparation of Cyclic 2', 3'-*O*-Isopropylidene Adenosine Diphosphate Ribose (115, cAcetDPR)

NAcetD$^+$ (**105**, triethylammonium salt, 7.5μmol, contaminated with 2′, 3′-*O*-isopropylidene (5′-Omethoxy)monophosphate) (**112**), was cyclised as described (section 7.5.1) and the product cAcetDPR (**115**), which eluted between 150 and 170mM TEAB, was evaporated to dryness *in vacuo* keeping the water bath temperature below 30°C.

Excess TEAB was removed by evaporation with MeOH and the cyclic compound was isolated as the glassy triethylammonium salt in 46% yield as quantified by UV and stored at -70°C.

NMR δ_H (400MHz, D_2O) 1.30 (3H, s, $OC(CH_3)_2$), 1.49 (3H, s, $OC(CH_3)_2$), 3.80-3.84 (1H, m, H_A5'), 3.95-3.96 (1H, m, H_A4'), 3.99-4.02 (1H, m, H_R5'), 4.24-4.27 (1H, m, H_R5'), 4.34-4.36 (1H, m, H_R3'), 4.43 (1H, m, H_A5'), 5.37 (1H, dd, J=3.1, 6.1Hz, H_A3'), 5.62 (1H, dd, J=2.0, 6.1Hz, H_A2'), 6.02 (1H, d, J=3.7Hz, H_R1'), 6.25 (1H, d, J=2.0Hz, H_A1'), 8.26 (1H, s, H_A8), 8.91 (1H, s, H_A2); other protons are obscured by the water peak at 4.8ppm,

δ_P (161.7MHz, D2O) -11.14, -11.52ppm (2d, J_{PP}=13.9Hz),

UV: λ_{max}=257nm, ε=14.3x10^{3}M^{-1}cm^{-1} (as for cADPR),

HPLC: retention time 7.1min,

m/z (FAB$^+$) 582 (M^++1); (FAB$^-$) 580 (M^+-1).

7.5.5 Preparation of Cyclic Acycloadenosine Diphosphate Ribose (116, cAcycloDPR)

NAcycloD$^+$ (**106**, triethylammonium salt, 1μmol) was cyclised as described (section 7.5.1) and the product cAcycloDPR (**116**), which eluted between 130 and 150mM TEAB, was evaporated to dryness *in vacuo* keeping the water bath temperature below 30°C. Excess TEAB was removed by evaporation with MeOH and the cyclic compound was isolated as the glassy triethylammonium salt in 21% yield as quantified by UV and stored at -70°C.

UV: λ_{max}=257nm, ε=14.3x10^{3}M^{-1}cm^{-1} (as for cADPR),

HPLC: retention time 5.1min,

m/z (FAB$^+$) 483(M^+), 192 (M^+-ribose-$P_2O_7H_2$).

7.5.6 Preparation of 7-Deaza Cyclic Adenosine Diphosphate Ribose (117, 7-Deaza-cADPR)

7-Deaza-NAD^+ (**107**, triethylammonium salt, 3.6μmol) was cyclised as described (section 7.5.1) and the product 7-deaza cADPR (**117**), which eluted between 90 and 110mM TEAB, was evaporated to dryness *in vacuo* keeping the water bath temperature below 30°C. Excess TEAB was removed by evaporation with MeOH and the cyclic compound was isolated as the glassy triethylammonium salt in 57% yield as quantified by UV and stored at -70°C.

NMR δ_H (400MHz, D_2O) 3.9-4.45 (6H, m, $2H_A5'$, $2H_R5'$, H_A4', H_R3'), 5.34 (1H, dd, J=5.6, 6.4Hz, H_A2'), 5.75 (1H, d, J=6.4Hz, H_A1'), 6.26 (1H, d, J=4.0Hz, H_R1'), 6.71 (1H, d, J=3.7Hz, H_A7), 7.31 (1H, d, J=3.7Hz, H_A8), 8.75 (1H, s, H_A2); other protons are obscured by the water peak at 4.8ppm,

δ_P (161.7MHz, D_2O) -12.7, -13.4ppm (2d, J_{PP}=14.4Hz),

UV: λ_{max}=272nm, ε=8.15x$10^3M^{-1}cm^{-1}$,

HPLC: retention time 4.87min,

m/z (FAB^+) 541 (M^++1), (FAB^-) 539 (M^+-1).

7.5.7 Preparation of 7-Deaza-8-Bromo Cyclic Adenosine Diphosphate Ribose (118, 7-Deaza-8-bromo-cADPR)

7-Deaza-8-bromo NAD^+ (**108**, triethylammonium salt, 12μmol)) was cyclised as described (section 7.5.1) and the product 7-deaza-8-bromo-cADPR (**118**), which eluted between 130 and 150mM TEAB, was evaporated to dryness *in vacuo* keeping the water bath temperature below 30°C. Excess TEAB was removed by evaporation with MeOH and the cyclic compound was isolated as the glassy triethylammonium salt in 31% yield as quantified by UV and stored at -70°C.

NMR δ_H (400MHz, D_2O); 3.89-3.93 (1H, m, H_A5'), 3.98-4.03 (1H, m, H_R5'), 4.15-4.20 (1H, m, H_A4'),4.23-4.27 (1H, m, H_R5'), 4.30-4.40 (2H, m, H_A5', H_R3'), 5.44 (1H, dd, J=5.2, 6.1Hz, H_A2'), 5.97 (1H, d, J=4.0Hz, H_R1'), 6.03 (1H, d, J=6.1Hz, H_A1'), 6.91 (1H, s, H_A7), 8.75 (1H, s, H_A2), all other protons are obscured by the water peak at 4.8ppm

δ_P (161.7MHz, D_2O); -10.8, -11.6ppm (2d, J_{PP}=18Hz),

UV: λ_{max}=277nm, ε=10.85x$10^3M^{-1}cm^{-1}$,

HPLC retention time 4.8min,

m/z (electrospray$^+$) 619, 621 (M^++1); (electrospray$^-$) 617, 619 (M^+-1), 79, 81 (Br^-).

7.5.8 Preparation of Cyclic Inosine Diphosphate Ribose (122, cIDPR)

A final volume of 2ml of a 1.9mM solution of NID^+ (**109**) in 25mM HEPES buffer at pH 6.8 was incubated with 50μl of crude *Aplysia* ADP-ribosyl cyclase at room temperature. The reaction was monitored by HPLC and was quenched by a twenty fold dilution of the reaction mixture with water when the peak attributable to nicotinamide was no longer

seen to be increasing, approximately 4h. The desired product was isolated from a small amount of unreacted starting material and the by-product nicotinamide using ion-exchange chromatography eluting with 0-75mM trifluoroacetic acid. The product, which eluted between 20 and 30mM trifluoroacetic acid, was evaporated to dryness *in vacuo* keeping the water bath temperature below 30°C. The cyclic compound was isolated in 75% yield as quantified by UV and stored at -70°C.

NMR δ_H (400MHz, D_2O); 3.90-4.60 (10H, m, H_I2', H_R2', H_I3', H_R3', H_I4', H_R4', $2H_I5'$, $2H_R5'$), 6.19 (1H, m, H_I1'), 6.34 (1H, m, H_R1'), 8.27 (1H, s, H_I2), 9.22 (1H, s, H_I8),

δ_P (161.7MHz, D_2O); -11.38ppm (brs),

UV: λ_{max}=254nm, ε=5x10^{3}M^{-1}cm^{-1},

Fluorescence: excitation 250-350nm, emission 410nm, λ_{max}=285nm

excitation 300nm, emission 350-560nm, λ_{max}=385nm,

HPLC: retention time 3.15min,

m/z (electrospray$^-$) 542 (M^+), 541 (M^+-1), 540 (M^+-2), 269 (M^+-ribose-$P_2O_7H_2$).

7.5.9 Preparation of Cyclic Guanosine Diphosphate Ribose (123, cGDPR)

NGD$^+$ (**121**, free acid, 15µmol) was cyclised as described (section 7.5.8) and the product cGDPR, (**123**) which eluted between 14 and 18mM trifluoroacetic acid, was evaporated to dryness *in vacuo* keeping the water bath temperature below 30°C. Excess TEAB was removed by evaporation with MeOH and the cyclic compound was isolated in 93% yield as quantified by UV and stored at -70°C.

NMR δ_H (400MHz, D_2O); 3.90-4.60 (10H, m, H_G2', H_R2', H_G3', H_R3', H_G4', H_R4', $2H_G5'$, $2H_R5'$), 5.97 (1H, m, H_G1'), 6.12 (1H, m, H_R1'), 8.89 (1H, s, H_G8),

δ_P (161.7MHz, D_2O); -11.40ppm (brs),

UV: λ_{max}=257nm, ε=8.76x$10^3M^{-1}cm^{-1}$,

Fluorescence: excitation 250-350nm, emission 410nm, λ_{max}=300nm

excitation 300nm, emission 350-560nm, λ_{max}=425nm,

HPLC: retention time 3.40min,

m/z (electrospray$^+$) 558 (M^++1); (electrospray$^-$) 557 (M^+), 556 (M^+-1), 555 (M^+-2), 277 (M^+-ribose-$P_2O_7H_2$).

7.6 Inhibition of Cyclase Activity

The Michaelis constant (K_m) of the true enzyme substrate NAD^+ and the inhibition constant (K_i) for nicotinamide benzamide dinucleotide (**111**) were calculated as outlined below. For these experiments all dilutions were made using 25mM HEPES solution which had been adjusted to pH=7.0 using 0.1M HCl. The term reaction refers to the mixing of the enzyme with the substrate regardless of any other components in the system.

The K_m of the natural substrate NAD^+ (**8**) for the cyclisation reaction with the *Aplysia* cyclase enzyme was determined as follows. Enzyme stock solution ($100\mu gml^{-1}$) was prepared and an aliquot (50µl) added to a volume of buffer (450µl-volume of NAD^+ solution). To this was added the appropriate volume of 1mM NAD^+ to give the final concentration of NAD^+ ([S]) in the assay solution as 7.5µM. One minute after mixing an aliquot (20µl) was removed and injected into the HPLC. This was repeated every minute for a further 6 minutes and then the full HPLC trace was allowed to develop for a further 4 minutes.

From the HPLC trace the peaks referring to the nicotinamide produced after a given time of the reaction were identified. The areas under these peaks, as integrated by the HPLC recorder, were then plotted against time (namely the time that the aliquot was removed from the sample) to give a time progress curve for the given concentration of substrate. Linear regression calculations were made of this plot and used to calculate the gradient of the line. The gradient itself was used as a measure of the initial velocity (v) of reaction

for this concentration of substrate. Analogous methods were used for 15, 50, 75 and 150μM assay concentrations of NAD^+.

A Lineweaver-Burk plot was obtained by plotting 1/v against $1/[S]_{t=0}$. The equation for the line is given below:

$$1/v = K_m/V_{max}\ 1/[S] + 1/V_{max}$$

The K_m for the enzymatic cyclisation reaction with NAD^+ as the substrate was therefore determined by extrapolating the plot until it intercepted the x-axis. The value of 1/[S] when 1/v=0 was equivalent to $-1/K_m$. From these calculations the K_m for the cyclisation of NAD^+ (**8**) to cADPR (**7**) using *Aplysia* cyclase was calculated to be 100μM.

The K_i of nicotinamide benzamide dinucleotide (**111**) was also determined in this manner by including a known concentration of the inhibitor with the assay solution. Stock concentrations were made such that the final volume of the assay solution was kept at 500μl. The concentrations of inhibitor used ([I]) were 0, 100, 200, 300, 600nM. For each of these concentrations of inhibitor (**111**) a time progress curve was obtained exactly as outlined above for the following concentrations of NAD^+ (**8**) 20, 100, 200, 300, 500μM. The gradients of the lines thus obtained were again taken to be the velocities of these reactions.

For a given inhibitor concentration 1/v was plotted against $1/[S]_{t=0}$ in the same manner as for the K_m and this was repeated for all the inhibitor concentrations. The five Lineweaver Burk plots were then superimposed onto the same axis and the point at which they intercepted with each other was an indication of the type of inhibition that was being

observed. For nicotinamide benzamide dinucleotide (**111**) the lines crossed when $1/[S]_{t=0}$ =0 which was indicative of competitive inhibition.

For a competitive inhibitor the following equation is true:

$$1/v = K_m/V_{max} (1+[I]/K_i) 1/[S]+ 1/V_{max}$$

This being the case the gradient of a Lineweaver Burk plot for a competitive inhibitor is now given by:

$$\text{Slope}_{1/[S]} = (K_m/V_{max} K_i) \times [I] + K_m/V_{max}$$

Hence a plot of the gradients of each of the five Lineweaver Burk plots vs [I] at which the gradient was obtained was a straight line. This graph was extrapolated until it intercepted the x-axis and the [I] when $\text{Slope}_{1/[S]}$ was equal to $-1/K_i$. For nicotinamide benzamide dinucleotide (**111**) the K_i was calculated to be 365nM.

7.7 Biological Testing of cADPR Analogues

7.7.1 Preparation of Homogenate

Sea urchin egg homogenate was prepared as described.[24] Briefly, eggs of *Lytechinus pictus* sea urchins were dejellied, washed once with artificial sea water and then with Ca^{2+} free sea water containing 1mM EGTA, twice with Ca^{2+} free sea water without EGTA and then once with an intracellular medium containing gluconate (10-15ml of each). The eggs were then resuspended in 1 volume (50% egg suspension) of buffered intracellular medium at pH 7.2, cooled to 4°C, homogenised and centrifuged and the supernatant was collected and stored at -70°C.

Before use the homogenate was defrosted and diluted to give a 2.5% egg suspension using a buffer containing an ATP regenerating system, mitochondrial inhibitors and protease inhibitors. Finally Fluo-3 fluorescent dye (3μM) was added and the homogenate was incubated at 17°C. Extra-microsomal Ca^{2+} was measured by monitoring Fluo-3 fluorescence (excitation 490nm and emission 535nm) in a Perkin-Elmer LS-50B fluorimeter.

7.7.2 Calcium Release Measurements

Homogenate (0.5ml) was placed in a cuvette and the test compound, which had also been dissolved in the same intracellular like medium as the homogenate, was added in aliquots (5μl). EGTA (10μM) was added to the test compound in order to sensitise the system to

very low Ca^{2+} release. Calibration of the assay homogenate by addition of known amounts of Ca^{2+} allowed estimations to be made of the amount of Ca^{2+} -released by the test compound.

When testing for Ca^{2+} -release in the presence of an antagonist the Ca^{2+} releasing agent was added 30s after the addition of the antagonist.

7.7.3 Monitoring the Metabolism of the Analogues

Degradation of the compounds in egg homogenate was monitored as a function of their sustained Ca^{2+} -releasing ability in the assay system above. A solution of the test compound (500nM) was incubated with homogenate at 17°C and at time intervals an aliquot (50µl) was removed. This was assayed for Ca^{2+}-release, and therefore remaining cyclic compound, by addition to a further aliquot of assay homogenate (500µl). The assay homogenate was pre-treated with caffeine (1mM) to increase the sensitivity of the bioassay by approximately 10-fold so that Ca^{2+} - release could still be detected even when only low concentrations of cyclic compound remained.

7.7.4 Determination of [^{3}H]-cADPR binding.

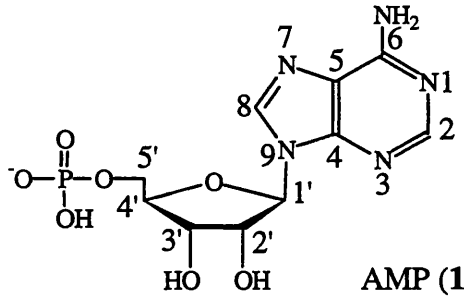
[^{3}H]-cADPR binding was determined in sea-urchin homogenates as described by Genazzani *et al.*.[281] In brief, homogenates were diluted in Glu-IM containing 1mM EGTA to a concentration of 1 mg protein / ml (approximately 3.5%) and incubated with 20 nM [^{3}H]-cADPR for 10min at 4 °C. Non-specific binding was assessed with 1 µM cADPR.

Binding was terminated by filtration (fiberglass GF/B filters) under vacuum and rapidly washed twice with ice-cold Glu-IM. Filters were dissolved in 14ml scintillation fluid and radioactivity was determined by liquid scintillation counting.

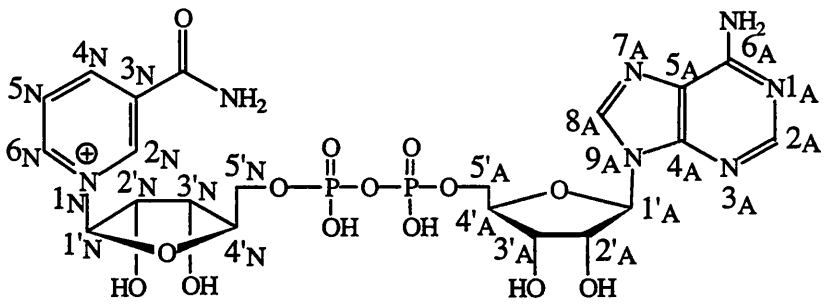
APPENDIX

Numbering of compounds

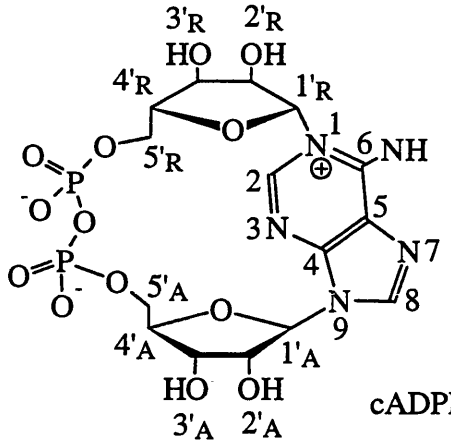
The compounds in this thesis have been numbered as shown below and this system was chosen to refer to the systematic naming of the parent adenosine so that compounds can easily be referenced to other analogues of cADPR. This numbering system was no longer systematic when the compounds became 7-deaza-analogues. The systematic nomenclature for the 7-deazaadenine ring system is pyrrolo[2, 3-*d*]pyrimidine and this ring is correctly numbered in a different manner to adenine, also shown below. However, for ease of recognition, all the compounds in this thesis have been named retaining the numbering used for adenine and referring the compound to adenosine in each case. If the compounds had been numbered systematically the so called "7-position" would in fact be the 5-position of 7-deazaadenosine and the "8-position" would be the 6-position.



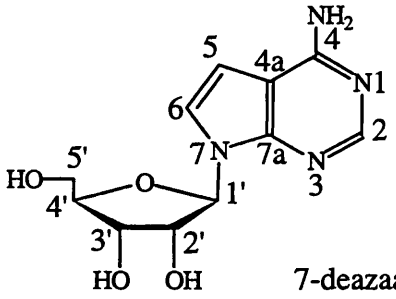
AMP (**19**)



NAD+ (**8**)



cADPR (**7**)



7-deazaadenosine (**58**)

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